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GROWTH OF ARTEMISIA ANNUA, ARTEMISININ SYNTHESIS, AND ARBUSCULAR MYCORRHIZAL FUNGUS COLONIZATION AS AFFECTED BY ACCESSION AND FERTILIZATION

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Abstract

Limited availability of artemisinin as a medicinal compound to overcome malaria disease is a serious problem that should be solved immediately. This research was designed to obtain combination of accession and fertilization to increase *Artemisia annua* L. biomass, artemisinin content, and arbuscular mycorrhizal fungus colonization. Experiment was conducted using Split Plot design with three replications. The main plot was the accession of *A. annua* L. which was green and purple stem. Subplot was inorganic/control and organic fertilizer (0, 0.5, 1, 1.5, and 2 ton ha^{-1} of compost). The experimental results showed accession and fertilization significantly interacted to increase plant height and biomass and AMF colonization. However, the levels of artemisinin and essential oils were affected by either accession or fertilization only. Inorganic fertilizer significantly increased plant height of *A. annua* at 4-8 weeks after planting. Conversely, the increase of plant biomass and AMF colonization was not affected by fertilization. Green and purple stem accession had the same plant height and biomass production. However, purple stem accession resulted in higher artemisinin contents and green stem accession had higher AMF colonization. *A. annua* L. purple stem accession cultivated with spacing 75 cm x 75 cm and compost at 0.5 t ha^{-1} was predicted to produce about 10 kg of artemisinin per hectare.

Keywords: *Arbuscular mycorrhizal fungus, Artemisia annua, artemisinin, organic fertilizer*

Introduction

It has been decades that human being had to struggle coping with malaria caused by *Plasmodium vivax, P. falciparum,* or *P. malariae*. Every year, about 500 million people are infected by *Plasmodium* and about one million of them died [1]. In Indonesia, malaria has infected more than half million people and caused death of 900 people annually [2]. A long term use of drug from quinine derivates has resulted in a number of *P. falciparum* strains more resistant to the drug.

World Health Organization (WHO) has recommended the use of artemisinin (sesquiterpene lactone) extracted from *Artemisia annua* L. as the substitute for quinine. The plant has been known by Chinese in 4th century as anti fever medicine [3,4]. However, coping malaria with artemisinin was hampered by limited supply of artemisinin in the world market [5]. In 2005, WHO predicted that drug for anti malaria containing artemisinin only 120 – 200 million dosage [2]. Similarly, artemisinin synthesis by chemical process or biotechnology is still low [6, 7]. Therefore, new strategy is required to improve artemisinin availability.

The improvement of *A. annua* L productivity in biomass and artemisinin by expanding growing area and intensification is short term strategy to improve artemisinin availability. Area expansion implies that *A. annua* is grown on area outside its natural habitat, but knowledge on the characteristics of habitat suitable for *A. annua* is limited [8]. Previous studies, [9] indicated that interaction between cytokinin application and dosage of N determined the biomass and artemisinin productions, whereas [10] reported otherwise. On nutrient deficiencies of Ultisol, biomass and artemisinin productivities were not affected by K application [11].

Intensification means improving biomass and artemisinin productivities per unit of land area. By improving plant biomass, higher availability of artemisinin can be expected, even the artemisinin content on each plant is not high. Arbuscular mycorrhizal fungus (AMF) colonization has been reported to improve secondary metabolite content, including artemisinin and aestheric oil contents in *A. annua* L. [12]. However, there was no sufficient information on AMF isolates compatible for *A. annua* L. under organic farming on acid mineral soil as in Bengkulu, Indonesia. For this reason, this study was undertaken to determine the best combination of *A. annua* L. accession and fertilizer application for biomass, artemisinin productions, and AMF root colonization of *A. annua* L.

Materials and Methods / Experimental

A field experiment was conducted from April to September 2012 at Pematang Donok, Kepahyang, Bengkulu (about 1000 m above sea level). Seeds of green and purple stem accessions of *A. annua* procured from The Medicinal Plants and Traditional Medicine Research and Development Institute (Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional, B2P2TO2T) Tawang Mangu, Central Java were germinated and maintained in nursery for two month. Transplanting of ten-leave plants was carried out on 3 m x 3 m plot according to split plot arrangement of the treatments laid on a randomized complete block design with three replications. *A. annua* accessions were allotted as the main plot and fertilizer applications, consisted of control, 0.5, 1, 1.5, and 2 ton compost ha^{-1} were allotted as the sub plot. The Control fertilizer was mixture of 100 kg N, 50 kg P_2O_5, and 50 kg K_2O ha^{-1}. Common cultural practices for raising healty plant were applied during experiment.

Observations were made on plant biomass (g, weight of oven dried plant at end of vegetative stage); N, P and K nutrient content of leaves (% dry weight) using general methods at Indonesian Soil Research Institute [13]; artemisinin and aestheric oil content of leaves (% dry weight, measured using Thin Layer Chromatography as developed by B2P2TO2OT); and AMF colonization on plant root measured using modified Phillip and Hayman's [14].

Collected data were subjected to analysis of variance on sample means. Means comparison was made using Duncan Multiple Range Test at 5% significant level. Correlation and regression analyses were performed to evaluate associations among observed variables.

Results and Discussion

The present study showed that there were no significant different between the accession of *A. annua* on leave N, P, and aestheric oil contents (Table 1). However, they were different on plant biomass, AMF colonization, and artemisinin content. Fertilizer had positive effects on plant height, plant biomass, AMF colonization, and artemisinin content. Each accession showed a different response to fertilizer, indicating the existence of interaction effect between accession and fertilizer application on plant height, plant biomass, and AMF colonization.

Tabel 1. Effects of accession and fertilizer application on Artemisia annua growth at eighth week after planting.

Variable	Accession (A)	Fertilizer application (P)	A x P interaction
Plant biomass	*	**	**
Percentage of AMF colonization	*	**	**
N-content of leaves	ns	ns	ns
P-content of leaves	ns	ns	ns
Arhtemicyne content	*	*	ns
Aestheric oil content	ns	ns	ns

Note: ns = not significantly different ($p > 0.05$), * = significantly differeny ($p \leq 0.05$), ** = highly significant different ($p \leq 0.01$)

Plant biomass was significantly affected by interaction between accession and fertilizer application (Table 1). This indicated that effect of fertilizer was determined by accession of *A annua*. Organic fertilizer showed better effect on plant biomass than inorganic fertilizer. On average, organic fertilizer produced 75.66 g per plant biomass which was higher than inorganic fertilizer (61.13 g) (Table 2). The highest plant biomass (121.62 g) was produced by purple-stem accession with compost application at 0.5 ton ha^{-1}. Although the plants received this treatment was not as tall as thosed received inorganic fertilizer, they had more branches and leaves. In addition, the plants performed more vigorous compared to the other treatments.

Table 2. Mean plant height at 4 to 8 week after planting, plant biomass, and AMF colonization of *Artemisia annua* with different rate of fertilizer applications.

Accession	Fertilizer	Plant height (cm) at 4 to 8 after planting					Biomass (g/ plant)	AMF colonization (%)
		4	5	6	7	8		
Green	Inorganic	73.00 b	82.67 ab	105.67 b	123.33 b	136.67 bcd	51.48 d	67 a
Green	Compost 0.5t ha^{-1}	73.33 b	82.00 ab	103.67 b	117.67 b	131.33 de	63.71 bcd	70 a
Green	Compost 1.0t ha^{-1}	74.00 b	85.00 ab	107.67 b	125.67 b	146.33 b	56.51 cd	50 a
Green	Compost 1.5t ha^{-1}	77.67 ab	88.67 ab	112.67 b	125.67 b	140.67 bcd	73.17 bc	47 a
Green	Compost 2.0t ha^{-1}	74.00 b	82.33 ab	104.00 b	118.00 b	137.67 bcd	75.00 bc	47 a
Purple	Inorganic	87.67 a	96.33 a	129.00 a	143.00 a	176.33 a	70.79 bc	37 ab
Purple	Compost 0.5t ha^{-1}	71.33 b	81.00 ab	103.67 b	115.33 b	134.00 cd	121.62 a	43 a
Purple	Compost 1.0t ha^{-1}	59.00 c	65.00 c	82.67 c	98.67 c	121.00 e	63.88 bcd	33 ab
Purple	Compost 1.5t ha^{-1}	66.33 bc	75.33 bc	102.00 b	116.33 b	144.33 bc	69.77 bcd	0 b
Purple	Compost 2.0t ha^{-1}	76.67 ab	85.67 ab	110.00 b	126.33 b	148.33 b	81.60 b	30 ab
Inorganic vs Organic								
	Inorganic	80.33 a	89.50 a	117.33 a	133.17 a	156.50 a	61.13 b	52 a
	Organic	71.54 a	80.63 b	103.29 b	117.96 b	137.96 b	75.66 a	40 a

Mean in the same column followed by the same alphabet indicated non-significant on Duncan test at 5% level.
Inorganic fertilizer = 100 kg N + 50 kg P_2O_5 + 50 kg K_2O ha^{-1}

AMF colonization on both accessions was determined by fertilizer application (Table 1). Inorganic fertilizer produced 52 % colonization, which was comparable to average of organic fertilizer (40%)(Table 2). AMF colonization on purple-stem accession (56%) was higher than on green-stem accession (29%). Compost at 0.5% resulted in the highest AMF colonization, although it was statistically not significantly different with other treatments.

Artemisinin content was affected by accession and fertilizer application (Table 1). Purple-stem accession had artemisinin content (0.47%) which was significantly higher than green-stem accession (0.43%) (Fig. 1). Organic fertilizer produced higher artemisinin content than inorganic fertilizer. Among compost applications, 1.0 t ha^{-1} produced the highest artemisinin content. However, applications at 0.5 to 2.0 t ha^{-1} tended to produce artemisinin a slight increment from 0.48 % to 0.59%.

Besides producing the highest plant biomass, compost application at 0.5 t ha^{-1} on purple-stem accession also produced relatively high artemisinin content (0.48%)(Fig 1), which was higher compared to previous study [15]. Laboratory analysis showed that the soil used in this study was lack of N and K. The compost containing 0.91% total N could contribute N-content of soil, mainly NO_3^- that is required in plant tissues and artemisinin formations. However, dosage of application was apparently relative high resulting a non-significant effect on N-content of plant tissue (Table 3).

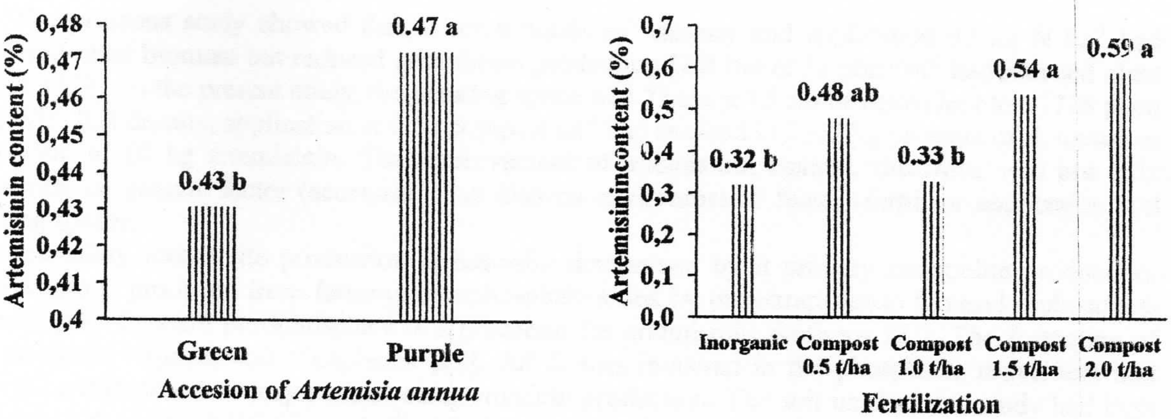

Fig. 1. Effect of accession (left) and fertilizer application (right) on artemisinin content of *Artemisia annua* L. at 8 week after planting.

Table 3. Mean N, P, aestheric oil contents of leaves tissue of *Artemisia annua* at 8 week after planting.

Accession	Fertilizer	N-content (%)	P-content (%)	Aestheric oil content (%)
Green	Inorganic	3.28	1.30	0.43
Green	Compost 0.5t ha^{-1}	3.82	1.36	0.43
Green	Compost 1.0t ha^{-1}	3.35	1.34	0.40
Green	Compost 1.5t ha^{-1}	3.98	1.81	0.43
Green	Compost 2.0t ha^{-1}	4.56	1.37	0.55
Purple	Inorganic	3.28	1.27	0.43
Purple	Compost 0.5t ha^{-1}	2.74	1.22	0.50
Purple	Compost 1.0t ha^{-1}	3.21	1.40	0.38
Purple	Compost 1.5t ha^{-1}	6.17	1.34	0.48
Purple	Compost 2.0t ha^{-1}	3.10	1.51	0.58

No significant different between accession and among fertilizer applications on N, P, and aestheric oil content.
Inorganic fertilizer = 100 kg N + 50 kg P_2O_5 + 50 kg K_2O ha^{-1}

Compost application improves not only on N-content of soil but also on carbon compound that positively affect artemisinin production [16]. However, the improvement of compost dosage had not significantly improve plant growth and artemisinin production. Dosage 0.5 t ha^{-1} or equivalent to 5 kg N ha^{-1} was sufficiently improve biomass production and artemisinin content in green-stem *A. annua* grown at Pematang Donok, Kepahyang, Bengkulu. Furthermore, increasing K resulted from improvement of compost dosage can reduce artemisinin content [11]. Compost used in this study had K-content that meet the national standard SNI 19-7030-2004. However due to lack of K-content in the soil, improvement in the dosage of compost application had no negative effect on artemisinin content.

Significant interaction effects between accession and fertilizer application on plant height, plant biomass, and AMF colonization indicated that genetic factor (accession) and environment (fertilizer application) played important roles in these variables. Similarly, artemisinin production in the present study was affected by accession (genetics) or fertilizer application (environment). This indicated that the effects of genetic and environmental factors were comparable but they unnecessarily produced significant interaction effect on plant growth and artemisinin production. In earlier research, some workers concluded that effect of genetic factor was stronger than environmental factor on plant growth and artemisinin production [17, 8]. Such hypothesis was argued by other workers that environmental factor, such as planting density [18], N and cytokinin applications [9], day length and N application [15], nitrate content, phosphate, carbon compound (sucrose or glucose) [16] and micro nutrients sufficiency [19] also had positive effects on plant growth and artemisinin production in *A. annua*.

The previous study showed that at three plants m^{-2} density and application 97 kg N ha^{-1} had improved plant biomass but reduced artemisinin production [20] but at 11 plant m^{-2} had reduced plant biomass [18]. In the present study, the planting space was 75 cm x 75 cm or equivalent to 17778 plant ha^{-1}. With this density, application at 0.5 t compost ha^{-1} had resulted in 2162 kg biomass of *A. annua* or equivalent to 10 kg artemisinin. The improvement of artemisinin content, therefore, was not only dependent on genetic factor (accession), but also on environmental factor (fertilizer application and planting space).

Secondary metabolite production is basically determined by it primary metabolite production. Artemisinin is produced from farsenyl pyrophosphate aided by preniltransferase farnesyl diphosphate enzyme [16]. Farsenil pyrophosphate is a precursor for artemisinin synthesis [21]. The formation of this precursor requires soil phosphates [22]. All factors involved in the phosphates movement and absorption therefore had major effect on artemisinin production. The soil used in this study had high available P_2O_5 content (18.03 mg kg^{-1}). On the other hand, compost studied containing 0.30% P and categorized as low according to SNI 19-7030-2004. This made soil factor affected P supply more than compost.

High soil P-content was suspected as the cause of non-significant effect of fertilizer application on AMF colonization (Table 2) and P-content of plant tissue (Table 3). P-content of soil was recoqnized as an inhibitor for formation and development of MA symbiosis [23]. Both accession of *A. annua* seemed to have different preference on AMF. AMF colonisation on green-stem accession (56%) was significantly higher than purple-stem accession (29%). However, it was reversed on artemisinin content where green-stem contained 0.43% artemisinin and purple-stem accession contained 0.56% artemisinin. This finding was inaccordance with previous studies that AMF inoculation could improve plant growth and artemisinin content [24, 25], however they did not mention the type of inoculants and AMF used in their study. Compatibility issue in AMF-plant symbiosis is commonly known [23]. Therefore, compatibility test should carried out to determine type of AMF that effective in improving biomass production and artemisinin content. The direct or indirect role of AMF on primary and secondary metabolite production was previously reported by other researcher [26]. AMF colonization also has been reported to have capability of improving salicylic acid [27] and jasmonic acid [28] which, in turn, improve artemisinin content in *A. annua* L.

Conclusion

Inorganic fertilizer at 100 kg N + 50 kg P_2O_5 + 50 kg K_2O ha^{-1} had positive effect in improving plant height of *A. annua*, whereas compost application at 0.5 t ha^{-1} produced the highest plant biomass (121.62 g) and artemisinin content (0.47%) on purple-stem accession. The artemisinin content produced by compost application at 0.5 t ha was as high as that produced by compost application at 2.0 t ha^{-1}. Colonization of AMF was more influenced by soil characteristics that rich in available P_2O_5 than compost application. Green-stem accession was more colonized by AMF than green-stem accession. Artemisinin content was determined by accession or fertilizer application. Purple-stem accession had artemisinin content higher thant green-stem accession. Identification and production of AMF inoculants need futher study to determine the most effective AMF.

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