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Biomass and Artemisinin Production of *Artemisia annua* L. on Several Altitudes

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***Abstract* — Increasing supply of *artemisinin* as a multipurpose medicinal compound, especially to cope with malaria, is a global problem that should be solved immediately. This research aimed to obtain high artemisinin production from *Artemisia annua* L. cultivated at a suitable altitude at Bengkulu Province of Indonesia. Experimental design used was a Split Plot in Randomized Complete Block Design with three replications, altitude (1000, 1100 and 1200 m above sea level altitudes each located at Sukasari, Bandung Baru, and Tangsi Duren, Kepahyang District Province Bengkulu of Indonesia) as main plot and bio-fertilizer application (control, *arbuscular mycorrhizal fungi*, and *phosphate solubilizer fungi*) as subplot. Each experimental unit received 10 ton ha^{-1} of cow-dung. Research result shows that altitude significantly affects *artemisinin*, essential oil, *mycorrhizal* and phosphate *solubilizer fungus* population, but not affect biomass production. Overall, *Artemisia annua* L. was successfully planted at the 1000 – 1200 m above sea level. Biomass and *artemisinin* production was higher compared to another experiment, i.e. 350 g per plant with 0.21 – 0.43% of artemisinin content.**

***Keywords* — artemisia annua; artemisinin; arbuscular mycorrhizal fungi; phosphate solubilizer fungi; elevation.**

I. INTRODUCTION

Malaria is a mosquito-borne infectious disease affecting humans and other animals caused by parasitic protozoans (a group of single-celled microorganisms) belonging to the Plasmodium type. Malaria causes symptoms that typically include fever, fatigue, vomiting, and headaches. Malaria is a major cause of human mortality and morbidity in tropical endemic countries worldwide. More than two million people per year in more than 100 countries are threatened by malaria caused by *Plasmodium vivax*, *P. falciparum*, and *P. malariae*. Annually, more than 500 million people are infected with malaria, and more than 1 million of them died. The countries of sub-Saharan Africa account for the majority of all malaria cases, with the remainder mostly concentrated in Brazil, Turkey, India, Afghanistan, Sri Lanka, Indonesia, Vietnam, Myanmar, Cambodia, Thailand, and China. The death toll is reported at 1.3 million people each year, mostly young children in sub-Saharan Africa (90%), Southeast Asia (7%) and the Eastern Mediterranean Region (2%). Approximately half of the Indonesian population living in malaria-endemic areas. About 17 from 30 provinces in Indonesia was an endemic area of malaria. The average malaria cases has reached 5 : 1000 per annum and in high endemic regions has been reported to be as high as 876 peoples per 1000 peoples per annum [1], [2].

The emergence of strains of *Plasmodium falciparum* which resistant to anti-malarial drugs derived from *chinconas* making it more difficult for the control of malaria around the world. Therefore, the World Health Organization recommended the use of *artemisinin* combination therapies (ACT) as standard treatment worldwide for *P. falciparum* malaria. *Artemisinin* or *sesquiterpene lactone endoperoxide* is one of an effective anti-malarial compound and does not cause resistance to *Plasmodium* [2]. This peroxide is believed to be responsible for the drug's mechanism of action. Few other natural compounds with such a peroxide bridge are also known.

Artemisinin has been known and derived from *Artemisia annua* L. which is a herbaceous annuals plant that for centuries used as anti-fever in mainland China, known as *qinghaosu*, and proved to contain a compound namely

artemisinin [3]. This plant then spread to Africa, Europe and America and cultivated in many countries, for example in India, Vietnam, Thailand, Myanmar, Madagascar, Malaysia, United States, Brazil, Australia, and other countries in Africa and Europe.

Indonesia has a local clone that has not been cultivated, namely *A. papuana* which has *artemisinin* levels much lower than *A. annua* L. Therefore *A. annua* L. then introduced to Indonesia and known as *anuma*, but was used as a source of essential oils and not as a source of essential anti-malarial drugs. Generally, *A. annua* L. growing in Indonesia containing *artemisinin* as much as 0.22 to 0.40% depending on the method of extraction [4]

World Health Organization (WHO) and the United Nation Development Program (UNDP) was recommended the use of *artemisinin* for combating malaria diseases. Due to the recommendation of the WHO and UNDP on the use of *artemisinin* caused sharply increase of *artemisinin* demand. Low bioavailability of *artemisinin* in the *A. annua* L. plant and poor pharmacokinetic properties makes this molecule expensive and challenging to produce in order to meet the current worldwide demand, especially for economically disadvantaged people in developing countries. Synthesize *artemisinin* through biotechnological approaches is still far from expectations and yet economically infeasible [5] – [7]. So it takes a reasonably long time to get good results through the approach of modern biotechnology. Therefore, it is necessary to find several alternatives to improve the bioavailability of *artemisinin*.

One alternative to increasing the production of biomass of *A. annua* L. is cultivation improvement and expansion of planting area. Even the level of secondary metabolites in plants is a small amount but if it is produced on a large scale may be increasing the economic value of the products [8]. Researchers reported that organic fertilizer with a rate of 0.5 t ha^{-1} could produce biomass (121.62 g per plant) and artemisinin content (0.47%) on the accession purple stem *A. annua* L. plant [9], [10]. Accession purple stem artemisinin levels are significantly higher than green stem accession. However, higher root colonization by arbuscular mycorrhizal fungi (AMF) was found in green stem accession compared to purple stem accession. Other researchers have reported a link between phosphate levels and AMF activity with the induction of secondary metabolites associated with high levels of artemisinin [11] – [13].

AMF is members of the Glomeromycota and was important of soil microorganisms which help 80% terrestrial plant species including the majority of medicinal plants to combat some biotic and abiotic stresses [14], [15]. AM fungi have been shown to benefit crop productivity due to their contribution to plant nutrition particularly to increase phosphorus absorption from problem soils, soil structure, improving plant growth after an attack by pathogens and insects and other ecosystem services. It has been estimated that inoculation with AMF might result in a reduction of approximately 80% of the recommended fertilizer P rates under certain conditions

The production of *artemisinin* is a product of the plant biomass and *artemisinin* (%) content. High production of *artemisinin* can be obtained not only because of high levels of *artemisinin*. Low levels of *artemisinin*, if combined with high biomass, will also be able to produce high total production of *artemisinin*. Effect of AMF and other soil microorganisms for plant biomass improvement has known well. Some of AMF species and phosphate solubilizing microorganisms was reported compatible and made synergy with *Artemisia annua* L. and suggested the use of this microbial consortium for enhancing growth and the content and yield of *artemisinin* [16].

Researchers from Indonesia also reported that the highest levels of *artemisinin* (0.53%) produced by *A. annua* L. which was inoculated with AMF isolated from *A. annua L. rhizosphere* and its combination with the phosphate-solubilizing fungi (PSF) [9], [10]. AMF or PSF as a single inoculant yielded the highest biomass *artemisinin* but not the highest level of *artemisinin*. On the contrary, the combination of AMF and PSF produced high *artemisinin* content but they did not produce the highest biomass. *Artemisinin* produced on the research by [9] and [10] was higher when compared to previous studies that produced only 0.25 to 0.33% [17] or a range of levels of *artemisinin* (0.22 to 0.40%) *A. annua* L. plants growing in Indonesia [4].

The use of PSF is a promising biotechnological strategy in the management of phosphorus (P) fertilization, as it enables the recovery of P fixed in soil particles that cannot be utilized by plant root. The important genera of (PSF) are *Aspergillus* and some species of *Penicillium*. PSF does not lose the P dissolving activity upon repeated subculturing under laboratory conditions

However, there is no information of biofertilizer advantages will be obtained when *A. annua* L. is planted to different altitudes. Therefore, it is necessary to do testing of biofertilizer usages on the of several altitudes which still in the limit that can be tolerated by *A. annua* L. plants i.e. 1000 – 1200 m above sea level (asl).

II. Materials and Methods

The research was conducted in three villages, namely Tangsi Duren, Suka Sari and Bandung Baru located in Kabawetan, Kepahyang District, Bengkulu Province, Indonesia. The altitudes of the three villages are 1000, 1100, and 1200 m above sea level. Inoculant of AMF and PSF was produced by Laboratory of Soil Biology, Faculty of Agriculture, University of Bengkulu, Bengkulu, Indonesia. Measurement of *artemisinin* and essential oil contents was conducted in the Centre for Research and Development of Medicinal Plants and Traditional Medicine (B2P2TOOT), Tawangmangu, Karanganyar district, Central Java Province, Indonesia.

Seeds of accession purple stems of *A. annua* L. obtained from B2P2TO2T. AMF and PSF were isolated from *A. annua L. rhizosphere* grown at the sites. *Cow-dung manure* obtained from residents around the site of the experiment. Land in the experimental field was classified as fertile soil with high levels of *phosphate* but other nutrients were deficient (Table I).

TABLE I
CHARACTERISTICS OF SOIL FROM TANGSI DUREN, SUKASARI, AND BANDUNG BARU VILLAGES

Soil Characteristics	Tangsi Duren		Suka Sari		Bandung Baru	
Soil Texture	Sandy loam		Sandy loam		Sandy loam	
Sand	70.82		69.36		71.50	
Silt	10.65		12.61		12.66	
Clay	18.54		18.03		15.84	
pH H_2O	6.40	Neutral	6.50	Neutral	6.50	Neutral
C (%)	3.16	High	2.97	Medium	2.45	Medium
N (%)	0.20	Medium	0.24	Medium	0.26	Medium
P_2O_5 (mg kg^{-1})	19.00	Very high	18.45	Very high	18.33	Very high
Cation Exchange						
K [cmol(+) kg^{-1}]	0.67	High	0.52	Medium	0.54	Medium
Ca [cmol(+) kg^{-1}]	8.07	Medium	9.60	Medium	4.44	Low
Mg [cmol(+) kg^{-1}]	1.61	Medium	1.54	Medium	0.73	Low
Al [cmol(+) kg^{-1}]	0.16	Low	1.12	Low	0.14	Low
H [cmol(+) kg^{-1}]	0.12	Low	0.10	Low	0.10	Low
CEC [cmol(+) kg^{-1}]	21.38	Medium	24.23	Medium	22.43	Medium

Note: Classification of soil analysis results classified according to Soil Research Institute [18].

Seeds of kudzu plants (*Pueraria phaseoloides var. Javanica*) were germinated in plastic tubes containing zeolite that had been sterilized. Seeds of leaf two were inoculated by spores AMF isolated from the rhizosphere of purple stem accession *A. annua L.* plant. Seeds with *mycorrhizal* then embedded in plastic pots containing ± 100 g moist zeolite. Each plastic pot was then put in line the 2 m x 0.5 m x 0.5 (L x W x T) wooden shelves. Watering and fertilizing of low P level soil were given at a dose of 0.5 g per liter every three days. Plants maintained until the age of 2 months and then left to dry along with the medium. The top of the plant was then cut at the root collar. Growing medium mixed with roots and then used as an inoculant AMF. Inoculant was then stored in the shade and ready for the next step of the study.

The following procedure obtained inoculant of phosphate-solubilizing fungi (PSF). The soil of *A. annua L. rhizosphere* was taken composites. Soil samples measuring 0.5 mm diametre diluted with distilled water with a dilution series of 10, i.e., 10 g soil samples were suspended in 90 mL of distilled water using a sterile test tube so make 10^{-1} dilution. Ten mL suspension of a 10^{-1} dilution of soil was transferred to a test tube and added with 90 mL of sterile distilled water to obtain a dilution series of 10^{-2} and so on to obtain the dilution of 10^{-8}. A total of 0.2 mL suspension of last dilution series were transferred to Petri dishes containing gel-sprout media. Petri dish and its contents were rocked to homogenize and incubated at room temperature. After 2-3 days of grown fungal isolates were separated to obtain pure isolates. Isolates were then tested the ability to dissolve phosphate. Isolates that produced the widest halo zone were isolates of PSF that would be used. The isolate was then reproduced using the Pikovskaya media on test tube which put at a side in a wooden rack. Pure isolates were then taken and mixed material (carrier) in the form of rice bran and ready to be used as an inoculant PSF.

Four weeks old of *A. annua* L. seedlings were transplanted in the field with a spacing of 75 cm x 75 cm on the ground. All treatment was given by 10 t ha^{-1} of cow dung Placed in the planting hole ± 10 g inoculant AMF and 0.25 g of PSF inoculant. Maintenance was done by watering, replanting, and weeding mechanically.

The dry weight was measured from the sample taken at the end of the vegetative phase, namely when ± 25% of plants were flowering. Harvesting was done at 3 months after planting in the field. The parameters observed plant height, dry weight of plants (stalks and leaves), the levels of plant *phosphorus*, *artemisinin* and essential oils. Plant height was measured every two weeks to measure the height of the plants starting from the root collar until the last growing point.

HPLC measured artemisinin levels. A total of 100 mg of dry powder plants sieved with No. 40 (325 mesh) strain extracted with 10 mL of hexane using ultrasonic cleaner at 40 ° C for 15 minutes. Extracted material removed from the cleaner and left to stand for 30 minutes at room temperature to form a precipitate. A total of 1.2 mL clear section was later centrifuged at 10,000 revolutions per minute for 5 (five) minutes. A total of 3 mL of the supernatant was spotted on a stationary silica phase GF254 10 x 20 cm. Standard solution spotted with a volume of 0.5, 1.0, 1.5, 2.0, and 2.5 mL (0.05, 0.10, 0.15, 0.20 and 0.25 mg/spot). Eluate material was prepared by mixing 15 mL ethyl acetate with 35 mL of hexane and then inserted into a chromatography. The filter paper was dipped into a chromatography until saturated. Thin Layer Chromatography (TLC) plant was inserted and waited until the mobile phase travels a distance of 8 cm from the starting spotted point. The plates were removed and allowed to dry and then sprayed with a mixture of acetic acid + sulfuric acid + 4-*methoxy benzaldehyde* with a ratio of 50:1:50 and then heated at 105 ° C for 15 minutes. Profile chromatography *artemisinin* appeared at Rf 0:43 to red to purplish-red color that was readable by TLC Scanner 3 in λ max 540 nm. Levels of *artemisinin* was then calculated using a standard curve equation *artemisinin (Artemisinin* levels vs spacious spot).

A total of 20 g powder of *A.annua L.* was mixed with 500 mL of distilled water in the distillation flask *Casia* Indonesia. A little water was dripped into the trap. Flask was then heated at a rate of 30 drops per minute distillation for 6-7 hours. Distillation was stopped after boiling, and no additional volume observed. Flask was cooled at room temperature until the oil layer was visible. The volume of oil was measured with 0.01 mL accuracy. Oil content (volume/weight) could be calculated based on the proportion of the volume of oil by dry weight of the sample analyzed.

The design used was a split plot design in a Randomized Complete Block Design with three replications. The altitudes of research location (Tangsi Duren – 1000 m, Bandung Baru – 1100 m, and Sukasari – 1200 m above sea level) used as the main plot. The subplots were (1) control without inoculation of bio fertilizer, (2) isolates of AMF (P) taken

from purple stem accession *Artemisia annua L. rhizosphere*, and (3) inoculant of phosphate-solubilizing fungi (PSF).

III. Result and Discussion

This experiment proved that only altitudes or location factors that influenced the *artemisinin* content and essential oil, the soil pH, AMF and PSF population (Table II). Inoculation of biofertilizer has no effect on all parameters observed. This was different from reported before [9][10]. This indicates that the *A. annua L.* plant basically can be grown at an altitude of 1000 - 1200 m above sea level. However, altitudes did not form a clear pattern of influence on the levels of *artemisinin*. Altitudes 1000 m and 1200 m had the same effect but differently from 1100 m altitude.

Artemisin levels and essential oil produced by the *A. annua L.* plant which planted at an altitude of 1000 m and 1200 m turned out to be not significant (Table II). Levels of *artemisinin* and essential oil produced by the *A. annua L.* plant which planted on an altitude of 1100 m were lower than those grown at an altitude of 1200 and 1000 m. It shows that the altitudes have no real effect on levels of *artemisinin* and essential oil. Differences in other factors caused different levels of *artemisinin* and the essential oil.

High levels of *artemisinin* and essential oil were produced by *A. annua L.* grown on soil with high phosphate-solubilizing fungi population located in Sukasari and Tangsi Duren (Table II). *Artemisia annua L.* plants that grown in the three locations studied had the same total P content in plant tissue (Table II). This was due to the three locations had roughly the same characteristics; in particular, the levels of available P was all high (Table 1). It shows that there is no link between levels of soil P and P plant tissue with high levels of *artemisinin* and essential oil. *Mycorrhizal* fungi and phosphate-solubilizing fungi thus do not contribute to the P-uptake. This results significantly different with research results reported before [11 – 13]. Roles of AMF and PSF in this situation was unpredictable. Other researchers have reported a link between phosphate levels and AMF activity with the induction of secondary metabolites associated with high levels of *artemisinin* [11] – [13]. Performance of plant height grown in three experiment locations also showed similarity (Figure I). Plants were grown in Sukasari and Tangsi Duren generally were higher than those grown in Bandung Baru. However, the average dry weight produced by plants grown in Bandung Baru was similar to that produced in two other locations. The growth of *A. annua L.* in Bandung Baru looked lusher than those in Sukasari and Tangsi Duren.

Biomass produced, if compared with the results of previous studies [6], [7] showed a significant increase. Plant dry weight was usually only about 30-50 g per plant had increased up to more than 300 g per plant. However, *artemisinin* levels remained relatively unchanged at around 0.40% but still higher than observed by other researchers.

TABLE II
INFLUENCE OF LOCATION/ALTITUDES AND BIOFERTILIZERS ON NUMBERS OF PLANT BRUNCHES; PLANT FRESH AND DRY WEIGHT; PLANT ARTEMISININ, ESSENTIAL OIL, PHOSPHORUS CONTENT AND SOIL PH, AMF AND PSF POPULATION OF 10 WEEKS AFTER TRANSPLANTING OF ARTEMISIA ANNUA L.

Location	Numbers of brunches per plant		Plant Fresh weight (g)		Plant Dry weight (g)		Plant *Artemisinin* content (%)		Plant Essential oil (%)		Plant phosphorus content (%)		Soil pH (H_2O)		Soil pH (KCl)		Soil AMF Population		Soil PSF Population	
Sukasari (1200 m)	35.39	a	620.22	a	361.76	a	0.40	a	0.60	a	0.13	a	6.73	a	5.70	a	96	b	433.17	a
Bandung Baru (1100 m)	41.78	a	636.67	a	372.01	a	0.21	b	0.42	b	0.13	a	6.19	b	5.20	b	341	a	203.37	b
Tangsi Duren (1000 m)	37.67	a	678.06	a	358.33	a	0.43	a	0.62	a	0.14	a	6.64	a	5.34	ab	331	a	583.35	b
Block	ns		ns		ns		ns		ns		ns		ns		ns		ns		ns	
Location/altitude	ns		ns		ns		*		*		ns		**		**		*		*	
Inoculation	ns		ns		ns		ns		ns		ns		ns		ns		ns		ns	
Inoculation * Loc	ns		ns		ns		ns		ns		ns		ns		ns		ns		ns	
CV (%)	14.7		35.6		29.5		24.2		27.7		12.7		4.3		2.1		25.4		12.7	

Note: ns = not significantly difference ($p > 0.05$), * = significantly difference ($p < 0.05$). Average in the same column followed by the same letter are not significantly different at 5% level of significance

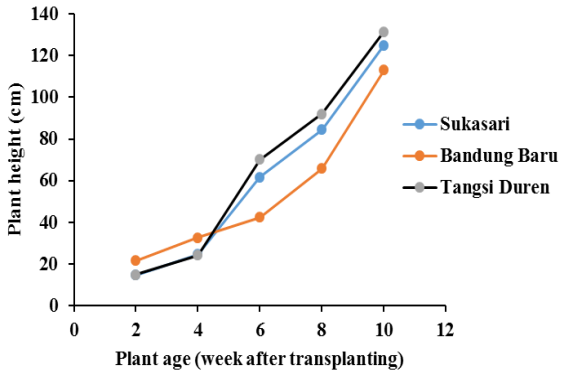

Fig. 1 Plant height of *Artemisia annua L.* at three research locations

High-level contents *of artemisinin* in *A. annua L.* plants that grown in the Bandung Baru and Sukasari seems closely related to the high population of PSF in the soil. The higher the PSF population is the higher level of *artemisinin* in *A. annua L.* plant. It is a bit difficult to conclude the relation of *artemisinin* level to the population of AMF in the soil. References [9] and [10] reported that the characteristics of the soil more influenced colonization of arbuscular mycorrhizal fungi compared to organic fertilizer. Green stem accession more colonized by *arbuscular mycorrhizal* fungi compared to purple stem accession. Reference [9] also reported that *artemisinin* levels determined by accession or fertilizer. *Artemisinin* level of purple stem accession was significantly higher than green stem accession. Inorganic fertilizers (100 kg N + 50 kg P_2O_5 + 50 K_2O kg ha^{-1}) had a positive influence on increasing of plant height of *A. annual* L. Otherwise, 0.5 t ha^{-1} of organic fertilizer can produce the highest level of biomass (121.62 g per plant) and *artemisinin* (0.47%) on the accession of purple. AMF isolated from purple accession of *Artemisia annua L. rhizosphere* combined with PSF or PSF alone could increase *artemisinin* production by 121% and 92% compared to the control-without the bio-fertilizer.

In this research, the altitude had a significant effect on the production of *artemisinin* and essential oil, soil pH, the population of soil AMF and PSF (Tables II). It showed that *A. annua* L. could be planted on altitudes of 1000 - 1200 m above sea level. So it is made the dry weight of the plants in this research was much more significant than the dry weight reported before [9], [10]. The dry weight was more than 350 g per plant even the levels of *artemisinin* only about 0.21 – 0.43%. Consequently, the total production of *artemisinin* had been increasing sharply compared with those produced by [9] and [10].

The lowest population of AMF found at the highest altitude but conversely for the PSF population. These findings were in accordant which reported by other researchers. Reference [19] suggested a decrease in *mycorrhizal* fungal diversity associated with plant roots with increasing elevation. Reference [20] reported that phosphate solubilizing microorganisms could tolerate a wide range of temperature and soil pH.

The production of *artemisinin* compound as a multipurpose medicine, especially anti-malaria, at the global level needs to be improved. One of the alternatives that can be done is by improving the cultivation of *A. annua L.* as producing *artemisinin.* There is an inspiring possibility that cultivation of *A. annua L.* can be improved in the humid tropics for commercial production, i.e., with good management practices and using the right accessions [21] and biofertilizer application particularly which help plant obtain unavailable phosphorus from soil [22], [23]. Through the use of biofertilizer is expected to achieve an increase in biomass production to levels sufficient *artemisinin.* Unfortunately, the theory that has been understanding by researchers and the results that have been obtained from laboratory and field trials so far are often widely apart. The problem is that the success of using biofertilizer is unpredictable. Different plant species often vary their response to the same biofertilizers. The success of using and biofertilizers persistence in the soil are affected by many factors, i.e. (i) species compatibility with the target plant and environment, (ii) the degree of spatial competition with other soil microorganisms, (iii) and the timing of inoculation. It is preferable to take these factors into account when inoculating biofertilizers to a target plant and environment in order to avoid failure of the inoculation process. The success technology is should be disseminated to the public so that people can be pro-actively nourish themselves. Also, people will also get new jobs, which is a producer of the biomass of *A. annua L.*

IV. Conclusions

Accession purple of *A. annua L.* can be grown at an altitude of 1000 - 1200 m above sea level and could produces more than 350 g per plant with *artemisinin* content about 0.21 – 0.43%. Biofertilizer did not affect biomass and *artemisinin* production due to a high level of soil phosphate or other unknown factors.

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