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Evaluation and Selection of Mutative Artemisia (*Artemisia annua* L.) According to the Altitude Variants

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Induction of genetic variant of *Artemisia annua* L. was conducted through the application of gamma ray irradiation in 2007-2008. The aim was to obtain a plant with high artemisine content $\geq$ 0.5% and late flowering period of about $\geq$ 7 month after planting. Tweleve selected genotypes were subsequently examined to gain genetic stability on altitude of 1500, 950, and 540 m asl. The results showed that the plants had shorter flowering age in Cicurug (540 m asl) than that of in Pacet (950 m asl) and Gunung Putri (1540 m asl). Genotype 8 had the latest age of flowering in the three locations than the other genotypes, however, the growth and biomass were the lowest. Vegetative growth of Artemisia in Pacet and Gunung Putri was better than those in Cicurug. Genotype of 15 in Cicurug and 5A genotype in Gunung Putri and Pacet had higher wet and dry weight than that of two other associates. Based on plant biomass, 5 genotypes from Gunung Putri and Pacet i.e. 1D, 3, 5A, 14, and 15 genotypes were selected, as well as 5 genotypes i.e. 1D, 3, 4, 5A, and 15 genotypes from Cicurug. Analisys on artemisin content successfully obtained 5 selected somaclone lines i.e. 1B, 2, 4, 14, and 3 somaclones.

Key words: *Artemisia annua*, mutation, genetic improvement, malaria, artemisinin

INTRODUCTION

Nowadays, malaria becomes one of the most threatening diseases in the world, menacing 300-500 milion people and kills more than one million people annually (Rao *et al*. 2006). *Quinine,* alcaloid extracted from Kinine bark was used and applied as medicine, in addition to the other medicine such as chloroquine and *sulphadoxine-pyrimethamine* (Greenwood & Mutabingwa 2002). The increasing resistance of the malaria parasite to the medicine, however, has caused the healing more complicated due to the incessant usage of kinine tablets for 20 years successively. For this reason, obtaining new medicine for malaria is deemed necessary (WHO 2004).

As medicinal herbs originated from China, *Artemisia annua* L. belongs to Asteraceae. It is a seasonal shrub (Mc Vaugh 1984). The plant is called *qinghao* in China and *sweet annie* or *worm wood* in America, and this plant was spread out in various countries such as Argentina, Bulgary, French, Hungary, Italy, Spain, and Yugoslavia (Klayman 1993).

The active substance of *Artemisia annua* L. in China is called *qinghaosu* or artemisinin. This substance is a lacton sesquterpene with Endoperoxide Bridge which is rarely found in nature. Clinically, this substance could inhibit the development of *Plasmodium* sp. Therefore, artemisinin is used for the active material for anti-malaria which is now available in the market. For centuries, Chinese have used *Artemisia annua* L. for malaria remedy (Ferreira *et al.* 2005).

The crucial problem of the Artemisia development in Indonesia is that the available genotype has a very low content of artemisinin, namely 0.01-0.5%. Therefore, in such level of content, Artemisia is neither effective nor economical to be developed in the industrial scale, China has succeeded in developing several clone with the artemisinin content > 1%. This clone however, is not spread out due to its specific location character. The other problem is the temperature. In the original place, this plant is planted in the altitude more than 1000 m. Meanwhile, such terrain in Indonesia is usually planted with vegetables. If we try to develop the plant in this area, there will be a conflict of interest.

Various approaches have been attempted to improve artemisinin production, such bioreactor of *A. annua* hairy root (Putalun *et al.* 2007), absisic acid (ABA) treatment (Jing *et al.* 2009), genetic engineering (Chen *et al.* 2000), and *in vitro* tissue culture (Liu *et al.* 2006). Indonesian Research Institute for Medicinal Crop (BALITRO) has carried out study on the artemisinin production and essential oil content enrichment through: genetic variation, clon adaptability examination, fertilizer as well

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as microbe application (Gusmaini & Nurhayati 2007), however, the result has not yet as it is expected.

Environmental factors, such as light (Wang *et al.* 2001), temperature (Guo *et al.* 2004), water and salt (Qian *et al.* 2007) greatly influence artemisinin content. In plant breeding, the mutant formation and *in vitro* culture will hopefully increase genetic variation of the plant; hence the selection could be conducted. New clones, therefore, should be obtained in order to find better artemisin content, in addition to the improvement and selection to the available lines. Until now, however, the expected result has not been gained. In the effort of prime clone provision, evaluation and selection to the obtained genotype is considered essential. Gamma-ray irradiation to the Artemisia shoot-tip has caused both qualitative and quantitative character variation (Purwati 2008).

This research was aimed to evaluate twelve selected artemisia genotypes from gamma-ray irradiation in order to obtain new genotype with higher biomass than that of the mother plant. And It is expected that the plant can do flowering latter than mother plant.

MATERIALS AND METHODS

This research was conducted in February-December 2009, in the three different altitudes of *experiment garden* on the Indonesian Research Institute for Medicinal Crops. They were Gunung Putri Cianjur (1540 m asl) and Cicurug Sukabumi (540 m asl) and Agricultural Biotechnology and Genetic Resources Research and Development Station at Pacet Cianjur (950 m asl).

Twelve Artemisia genotypes were selected from selected Artemisia somaclones obtained from mutation of gamma-ray irradiation i.e. 1B, 1C, 1D, 2, 3, 4, 5A, 6B, 7A, 8, 14, and 15 somaclones. They were selected based on wet weight, flowering age, and artemisinin content. Control was non-irradiated genotypes of the genotype from seed (CS) and *in vitro* (CI) production.

By using group-random experimental sampling, every treatment obtained three repetitive treatments and therefore in any location there were 42 experiment units consisting of five plants respectively. The observed variables were height, flowering age, wet and dry weight of the plants and artemisinin content.

Fertilizing was conducted twice, at the planting period and two month subsequently. The dosage was 3.5 g urea, KCl 1 g, and Sp18 3 g per plant.

RESULTS

At the second month after planting, the height average of the plants in Gunung Putri, pacet, and Cicurug showed significant result. The highest average height was obtained in Cicurug namely 150.6 cm and the lowest was that obtained in Gunung Putri, 93.57 cm. The observation result of the plant height in the respective experiment plot was as follows. The highest plant in Gunung Putri was 117.53 at the genotype 1D and the lowest was 57.4 of the genotype 8. The similar result occured in Pacet, where the plant height average was of the genotype 1C and 1D which became the highest plant, 197.53 cm and the genotype 8 showed the lowest height, 96.93 cm (Table 1).

The height of the plant during harvesting did not show different height from the mother plan, except that of genotype no 2. In Pacet, this genotype became the highest plant, 325 cm, compared to the control plant 261.3 cm. However, there was a tendency that genotype 1 D became the highest and genotype 8 became the lowest plant.

Flowering Age. Artemisia in Gunung Putri had latter flowering age than the one in Pacet and Cicurug. At the second month after planting at Gunung Putri, genotype 1D, 4 and CI had started flowering with the percentage 6.67, 20, and 13.33% respectively. Genotype 2, 4, 15, and CS genotype started flowering at the 3rd month after planting. The highest percentage of flowering plants at the 6th after planting was 51.9%. At the previous experiment (2008) for variety examination and somaclonal line selection, several somaclone lines which did not flowered at the 8th month after planting were obtained. This plant was 2 m high and had many branches; meanwhile the mother plant had flowered at the 7th month after planting .

In Pacet, the plant were flowered at the age of 4 months after planting. At the second month after planting, 8.1% has flowered, meanwhile at the age of 3 month after planting 24.29% has flowered and genotype 1B, 1C, 2, 4, and CI has the percentage of flowering more than 50%, meanwhile genotype 7A and 8 have not flowered at the same age as above (Table 2).

Artemisia in Cicurug flowered faster thant those in Pacet and in Gunung Putri. Genotype 1B, 1C, 2, 3, 14, and CI have flowering percentage more than 50%. At the 3rd month, almost all genotype has 100% flowered except the one, genotype 8, which has the flowering plants at the age of 4th month after planting. So the genotype 8 is the last to flower.

Table 1. Artemisia average height (cm) in three locations at the 2nd month after planting (MAP)

Genotype	Gunung Putri	Pacet	Cicurug	Average
1B	112.4ab	147.6bcde	161.0abc	140.3abc
1C	107.8abc	174.3a	165.1abc	149.0ab
1D	117.5a	174.3a	197.5a	163.0a
2	77.4e	128.5dcef	145.4c	117.1c
3	102.4abcd	157.2ab	183.6abc	147.7ab
4	77.8e	125.4ef	157.7bc	120.3bc
5A	89.0cde	149.9bc	188.7ab	142.5abc
6B	92.0cde	142.7bcde	159.8abc	131.5bc
7A	88.5cde	111.2fg	182.0abc	127.2bc
8	57.4f	101.0g	96.9d	85.1d
14	95.8bcde	126.1def	172.6abc	131.5bc
15	103.2abcd	150.6bc	187.0ab	146.9ab
CI	104.2abcd	148.7bcd	150.6bc	134.5abc
CB	84.0de	135.4bcde	165.1abc	128.3bc
Average	93.5C	140.9B	165.2A	133.2

Numbers followed with the capital number at the same row is not significantly different according to the further exam Duncan with the level 5%. Number followed with the similar alphabet at the same column is not significantly. Different according to the further exam Duncan with the level 5%. CI = *in vitro* control, CS = seed control

Table 2. Average percentage flowering plant of Artemisia in Gunung Putri, Pacet, and Cicurug (1, 2, 3, 4, 5, 6, 7 month)

Genotype	Month (%)																				
	1			2			3			4			5			6			7		
	G	P	C	G	P	C	G	P	C	G	P	C	G	P	C	G	P	C	G	P	C
1B	13.3	20.0	20.0	-	-	46.6	-	40.0	33.3	-	40.0	-	-	-		73.3			13.3		
1C	-	6.6	20.0	-	20.0	46.6	-	26.6	33.3	-	46.6	-	13.3	-		33.3			53.3		
1D	-	6.6	6.6	6.7	13.3	33.3	-	20.0	66.6	-	60.0	-	-	-		73.3			20.0		
2	-	-	-	-	6.6	60.0	6.7	60.0	40.0	6.7	26.6	-	6.7	6.6		73.3			-		
3	-	-	-	-	-	60.0	-	6.6	40.0	-	80.0	-	-	13.3		40.0			60.0		
4	-	-	-	20.0	33.3	40.0	6.7	33.3	60.0	-	20.0	-	-	13.3		33.3			40.0		
5A	-	-	-	-	-	46.6	-	6.6	53.3	-	73.3	-	-	20.0		40.0			60.0		
6B	-	-	-	-	6.67	40.0	-	40.0	60.0	-	46.6	-	-	6.6		80.0			13.3		
7A	-	-	-	-	-	46.6	-	-	53.3	-	53.3	-	6.7	46.6		73.3			26.7		
8	-	-	-	-	-	6.6	-	-	66.6	-	26.6	26.67	-	60.0		-			100		
14	-	-	-	-	-	53.3	-	13.3	46.6	-	86.6	-	-	-		40.0			60.0		
15	-	-	-	-	-	-	6.7	26.6	100.0	-	73.3	-	20.0	-		40.0			33.3		
CI	-	-	26.6	13.3	33.3	60.0	-	33.3	13.3	-	26.6	-	26.7	-		60.0			-		
CS	-	-		-	-	40.0	13.3	33.3	60.0	-	60.0		-	6.6		66.7			20.0		
average	0.9	3.3	5.2	2.86	8.1	41.4	2.38	24.2	51.9	0.48	51.4	1.90	5.24	12.3		51.9			35.7		

G = Gunung Putri, P = Pacet, C = Cicurug, CI = control *in vitro*, CS = control seed.

Harvesting. Based on the leaf and stem dry weight in the three experiment location, some genotypes shows higher production than that of the mother plant. In Gunung Putri at the height of 1540 m asl produced 902.59 kg while in Pacet 899.7kg and in Cicurug 183.04 kg. The result showed that the most productive plants were those planted in Gunung Putri at the altitude of 1540 m asl (Table 3 & 4). Compared to the mother plant originated from *in vitro*, it showed that the examined genotype produced more leaves and stems. Several somaclone lines producing sufficiently high biomass were genotype 1D, 3, 5A, 14, dan 15.

Artemisinin content at the examined plants were $\leq$ 0.5% in average. Compared to the mother plant, however, several genotype had higher content. In Cicurug in the 540 m asl, obtained 4 genotype containing $\geq$ 0.5% artemisinin is 1B, 2, 3, and 14 which has 0.63, 1.23, 0.62, and 0.59% respectively.

DISCUSSION

The plant growth in Gunung Putri, Pacet, and Cicurug at the second month after planting showed several varieties. Gentoype 1D was the highest to grow while genotype 8 was the lowest go ggrow. However, during the harvest, both plants did not show the significant difference. In fact, there was a tendency that genotype 1D was higher than the control plnat and other genotype. The variation of the mutated Artemisia has been observed at the prior experiment (2008) in Gunung Putri. The result of this observation has been used for early selection and evaluation in order to obtaine the expected genotype which potentially has higher production and artemisinin content. (Lestari *et al*. 2010). Variaton of the examined plant population showed some genotypes which can fulfill such expectation (Syamsudin *et al*. 1997).

The average height of the plants at the harvesting time in three location (Gunung Putri, Pacet, and Cicurug) reached more than two metres, it becomes phenomenon, therefore, that the Artemisia which is previously thought

Table 3. Wet weight leaves of artemisia in three locations planting (kg)

Genotype	Gunung Putri	Pacet	Cicurug	Average
1B	4625.3bc	4109.3cde	922.6bcd	3219.1bcde
1C	5534.0b	3680.6cde	723.8cd	3312.8bcde
1D	5698.7b	5398.7bcd	1622.2ab	4239.9b
2	4230.8bc	3209.7de	568.5cd	2669.7cde
3	5584.7b	6735.0ab	1058bcd	4459.2b
4	2604.8cd	2956.0e	1044.5bcd	2201.8de
5A	8250.7a	8286.7a	1318.9abc	5952.1a
6B	5160.4b	4326.8cde	830.4bcd	3439.2bcd
7A	4344.7bc	5598.7bc	1033.4bcd	3658.9bcd
8	1963.7d	3048.3e	707.6cd	1906.5e
14	5263.2b	5513.3bc	1013.4bcd	3930.0bc
15	5582.7b	4886.4bcde	2014.1a	4161.0b
CI	5275.3b	3232.7de	482.2d	2996.7bcde
CS	3952.7bcd	4983.0bcde	960.4bcd	3298.7bcde
Average	4862.3a	4711.8a	1021.4b	3531.83

Number followed with the similar alphabet at the same column is not significantly different according to the further exam Duncan with the level 5%. Numbers followed with the capital number at the same row is not significantly different according to the further exam Duncan with the level 5%.

Table 4. Dry weight of leaves of artemisia in three planting location (kg)

Genotype	Gunung Putri	Pacet	Cicurug	Average
1B	950.5bc	578.3bcd	139.5cd	556.1ab
1C	928.0bc	470.7bcd	126.0d	508.2b
1D	1171.0ab	851.7abc	313.4ab	778.7ab
2	880.2bcd	419.0cd	118.7d	472.6b
3	913.3bc	1069.7a	196.0bcd	726.4ab
4	538.3cd	463.8bcd	202.2bcd	401.5b
5A	1696.7a	1153.0a	270.5abc	1040.1a
6B	747.6bcd	551.2bcd	139.3cd	479.4b
7A	760.7bcd	879.6ab	191.8bcd	610.7ab
8	295.2d	492.2bcd	132.6cd	306.7b
14	1156.5ab	892.7ab	146.3cd	731.8ab
15	1065.3bc	812.8abc	350.2a	742.8ab
CI	892.7bc	341.0d	74.8d	436.2b
CS	640.3bcd	819.8abc	160.5cd	540.2ab
Average	902.59A	699.7A	183.0B	595.1

Number followed with the similar alphabet at the same column is not significantly different according to the further exam Duncan with the level 5%. Numbers followed with the capital number at the same row is not significantly different according to the further exam Duncan with the level 5%.

Table 5. Leaf dry weight and artemisinin content in selected genotype

Genotype in Gunung Putri (1540 m asl)	Leaf dry weight (kg)/artemisinin (%)	Genotype in Pacet (950 m asl)	Leaf dry weight (kg)/artemisinin (%)	Genotype in Cicurug (540 m asl)	Leaf dry weight (kg)/artemisinin (%)
1D	117.1 (0.34)	1D (0.34)	851.7	1B	313.4 (0.63)
3	913.3 (0.38)	3 (0.38)	1069.7	2	118.7 (1.23)
5A	1696.7 (0.43)	5A (0.31)	1153.0	3	196.0 (0.62)
14	1156.5 (0.22)	14 (0.42)	892.7	14	350.2 (0.59)
15	1065.3 (0.46)	15 (0.40)	812.8		

that because the plant is originated from the subtropical climate area which could not grow in the tropical area, now this plant could be planted even in the low level of tropical climate region.

Every location has its own age of flowering. In Cicurug artemisia flowered at the age of 2-3 month after planting. It is in accordance with the research by Ferreira *et al*. (2005) stating that Artemisia flower faster when planted in the low land, namely 75 days after planting. Artemisia planted in Pacet, flower after 3-4 month after planting. Meanwhile in Gunung Putri is at the age of 6-7 month after planting. Cicurug and Pacet with the height of 540 m dpl dan 950 m asl are less appropriate for developing Artemisia because the plant flowered fastly and therefore has the shorter vegetative phase, and therefore they produce low biomass.

Base on the leaf and stem dry weight, the plants in Gunung Putri showed the better growth than that of the other two places. Althought the artemisinin content is only $\leq$ 0.5%, when it was converted to biomass, the artimisinin content became higher. Several genotype in Cicurug produced artemisinin content of about $\geq$ 0.5% but it remained low when converted to biomass. It seemed that the agroclimate factor influenced the rate of artemisinin productivity. The required agroclimate factor will increase productivity and the secondary metabolite and vice versa. Ferreira *et al.* (2005) shows that genetic factor heavily influence artemisinin content.

Location significantly influences the Artemisia production (wet weight, dry weight, dry weight of leaves, and dry weight of stem). Plant biomas is also considerably influenced by the age of flowering. The longer the age of flowering, the higher of biomass could be produced because plant has vegetative period when the plant grow longer which in turn result in the biomass volume. Here the Artemisia planted in Gunung Putri has higher leaf wet weight and leaf dry weigh than the others which were planted in Pacet and Cicurug.

According to Namdeo *et al.* (2006), the artemisinin content in the leaves and flowers of artemisia was 0.01-1.1% from dry weight of the leaves and flowers, therefore in the Artemisia culture, the production was focused on the increasing leaves and flowers dry weight and artemisinin content. Artemisia planting in experiment Lembang (1.200 m asl) produced dry leaves 3 t/ha. Result conducted by Medicinal herbs Research Board in cooperation with Kimia Farma produced dry leaves as much as 5 t/ha.

Based on the leaf and stem dry weight and the artemisinin content, it can be concluded that Gunung Putri, at the height of 1540 m dpl was the best location for planting Artemisia. The adaptive genotype was 1D, 3, 5A,14, and 15. Even in Pacet, this genotype had the highest production although 1D, 3, and 4 had lower artemisinine than the control plant (Table 5). In Cicurug, the most adaptive genotypes are 1B, 2, 3, and 14. The result showed that there was such a genetic change of the examined genotype that they can adapted better than the mother plant. It was shown by their ability to produce higher biomass and artemisinine content than the mother plant. The yielded genetic variation produced abaca which is both the tolerant and moderate toward *Fusarium oxysporum* (Purwati *et al.* 2007), in addition, from the research has also obtained soybean tolerant to Polietilena Glycol selection (Widoretno *et al*. 2003).

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