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Research Article

MUTATION INDUCTION FOR IMPROVING OF ARTEMISININ CONTENT IN EACH PART OF *ARTEMISIA CINA* MUTAN LINES

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ABSTRACT

Mutation induction on *Artemisia cina* herbal medicine by gamma rays with the doses of 10, 20 and 40 Gy have been conducted at PAIR – BATAN. The purpose of mutation was to improve the plant traits which has more artemisinin content than control plant. The mutant lines interest were collected based on morphological characters (flowers, leaves, roots and stems). Artemisinin contents so far are only found in the leaves, therefore the improvement are expected not only to increase artemisinin content in leaves but also in the roots, stems and flowers of *A.cina* mutant lines were created by induction gamma rays by the doses of 10, 20 and 40 Gy. In every dose treatment was selected 8 mutant lines for analysis artemisinin content.To obtain artemisinin from all selected mutant lines, flowers, leaves, roots and stems were extracted by using n-hexan, and then fractionation by ethyl acetate. Artemisinin content were analyzed by High Pressure Liquid Chromatography (HPLC), and pure artemisinin used as standard. Mutation induction by the dose of 40 Gy improved dry weight of roots from 38.85 to 76.19g, dry weight of leaves of mutant lines from 10 Gy irradiation was twice higher than leaves of parent plant, and three times by the dose of 40 Gy, and no different at the dose of 20 Gy. The highest artemisinin content is 73.13mg/g in leaves of A26a3 mutant line and 36.68mg/g in flowers of A17.1 *A.cina* mutant lines, both of mutant lines were from mutation induction at the dose of 10 Gy. Mutation induction by the dose of 20 Gy could improve artemisinin content in stems part of B12.1 mutant line from non detection in parent plant to 0.83mg/g. Mutation induction at the dose of 40 Gy improved artemisinin content to 1.90mg/g in roots and 1.50mg/g in stems of C27b1 and C8.3 mutant lines respectively. *Artemisia cina* un-irradiated as parent plant only contain artemisinin in flowers, leaves and roots were 0.13; 0.45 and 0.05mg/g respectively. Dose of 40 Gy is the best dose for enhancement of artemisinin content in each part of *A.cina* mutant lines.

Key word : mutation, production of artemisinin, roots, stems, flowers

INTRODUCTION

In Indonesia, herbal medicines have been known since long time and up to the present the people still believed the useful of those plants. Indonesia is rich with many kinds of medicinal plants i.e *Typonium divaricatum* L. Decne (Aryanti, 2004), leaves of *Gynura procumbens* (Aryanti, *et al.*, 2007), and mungsi arab (*Artemisia cina*). *Artemisia cina* belong to the *Asteraceae* family as perenials and could grow at a latitude from 1000 to 1800m above sea level. Previous publication has mentioned as anti cancer and anti malarial capability of *A. cina* due to its artemisinin bioactive compound content.

The bioactive compound group in plants is found as alkaloid, steroid, flavonoid, and terpenoid, the activity of those compounds for anti imflamation, anti cancer, anti microbe have been studied and many papers were published. Flavonoid, saponins and tannins in bark, leaf, roots and stems of *Cyanthula prostates* (L) Blume was able to inhibit *Staphylococcus aereus* by minimum inhibition concentration (MIC) of 400µg/mL (Ogu *et al.,* 2012), and methanol extract of bark, leaf, roots and stem of *Ficus*

exasperate also able to inhibit the human pathogenic organism (Adebayo, *et al.,* 2013). Terpenoid like 1,8-cinnole and davone in *Artemisia abrotanum* is active as anti microbial agents (Brodin *et al.,* 2007). Another compound of terpenoid is artemisinin which is also found in *Artemisia cina*, this compound give the specific aroma in leaves and flowers of the plant. Plants with high artemisinin content could be identified with strong aroma from the leaves or flowers, and numbers of trichome at the back side of leaves which is also one of the characters of its high artemisinin content (Ferreira and Janicks, 1995).

Artemisinin has been used as malaria medicine combined with another drug and is famous Artemisinin Combine Therapy (ACT). Artemisinin obtained from plant is very low. For this reason, mutation breeding by using gamma rays or mutation induction is a right step for increasing artemisinin content in each part of *A.cina* plant.

Induced mutation is one of technique for plant breeding by using gamma rays as physical mutagen and could be used for genetic variation of plants. Centre for Isotopes and Radiation Application (CIRA/PAIR, BATAN) have released 20 new rice varieties by this mutation technique. Induced physical mutagenesis of gamma rays have been studied to *Amorphophallus muelleri* Blume by Santosa *et al* (2014) and applied to *Chataranthus roseus* (Syukur, 2000) and *Whitania somnifera* (L) Dunal by Bharathi *et al* (2014). Low doses of gamma irradiation is able to induce physiological and biochemical changes resulting in faster vegetative growth and early flowering (Berezina and Kaushankii, 1989). Heavy-ion beam mutation also have been used to create new varieties of *Artemisia annua* (Inthima *et al.,* 2014).

From the previous publication, artemisinin content was 61.66ppm in hairy roots of *A.cina* as a result of transformation by *Agrobacterium rhizogenes* bacteria, meanwhile artemisinin content of callus culture is 5mg/g (Aryanti, 2001 and Aryanti, 2010). Obaineh *et al* (2013) reported that bark of *Azadirachta indica* contain alcaloid 6.54mg/g, in leaf 53.2mg/g and roots 3-88 to 24.94%. This paper is the result of research on increasing artemisinin content in roots, stems, leaves and flowers of mutant lines of *A.cina* by mutation induction using doses of 10, 20 and 40 Gy. The purpose of this experiment is to obtain roots, leaves, stems and flowers of mutant lines with higher artemisinin content than that of control plant.

MATERIAL AND METHODS

Mutant lines of *Artemisia cina*

Mutation induction at the doses of 10, 20 and 40 Gy have been applied on buds of *A.cina in vitro*, two hundreds buds were irradiated for each dose in the Irradiator Panoramic Serba Guna (IRPASENA), at the Center for Isotopes and Radiation Application (CIRA/PAIR), National Nuclear Energy Agency (NNEA/ BATAN), Jakarta, Indonesia. The doses used in this experiment are below the lethal dose which 60 Gy. During *in vitro* culture, subculture and selection have been done based on agronomic traits like number of branch, wide of leaves, height and performance of plants.

The acclimatization was carried out in the green house and followed by cultivation in the field. Selected mutant lines were cultivated with a distance of 0.25 x1m in the filed. The size of the experiment plot was 4x5m for each number of mutant line and needed 240 buds for three replication.

Selection of mutant lines based on the performance and agronomic traits have also been done in the field. Selected mutant lines were harvested after 4 months of cultivation. Eight numbers of mutant lines were collected based on leaves area, number of leaves per plant, and plant height. Every mutant line is needed 30 plants to obtain the average of dry weight of flowers, leaves, roots and stems for determination of artemisinin content.

Artemisinin analysis

Each plant part (flowers, leaves, roots and stems) of the mutant plant and control plant were separated and dried at ambient temperature. Dried flowers, leaves, roots and stems were then weigh and blended. Extraction of each part used n-hexane. Ethyl acetate have been used for the fractionation using Sohly modified method.

Table I. Artemisinin content of roots, stems, flowers and leaves of *A. cina* mutant lines by the doses of 10, 20 and 40 Gy gamma rays

Doses (Gy)	Mutant lines	Roots (mg/g)	Stems (mg/g)	Flowers (mg/g)	Leaves (mg/g)
10	A6a1	ND	ND	9.46	ND
	A10.2	ND	ND	0.28	1.22
	A11.3	ND	ND	-	12.24
	A17.1	0.08	0.05	38.68	16.95
	A17.2	ND	ND	-	0.19
	A19a1	ND	ND	-	0.30
	A21.3	0.03	ND	ND	4.22
	A26a3	0.08	0.35	-	73.13
20	B1a2	ND	ND	-	0.05
	B7.2	ND	ND	-	0.04
	B10.2	ND	ND	-	0.04
	B12.1	0.44	0.83	-	2.24
	B16.2	0.25	ND	-	0.12
	B28.2	0.03	ND	ND	ND
	B24a2	ND	ND	0.12	0.08
	B24b3	0.100	ND	ND	0.15
40	C8.3	1.73	1.50	-	2.42
	C11.5	1.22	0.15	0.20	1.75
	C11b2	ND	ND	-	1.50
	C11b3	0.07	ND	0.02	0.12
	C15a2	1.64	ND	ND	0.20
	C18.2	0.05	ND	0.02	0.98
	C27a1	ND	ND	0.03	2.50
	C27b1	1.90	0.04	-	36.94
Parent plant		0.05	ND	0.13	0.45

Note : - No flower, ND (non detection)

Ethyl acetate fraction of each part of the number of mutant lines were analysis using Shimadzu High Pressure Liquid Chromatography (HPLC), with acetonitrile/ddwater (7/3) as eluent and μBondapak for column separation. Pure artemisinin was used for standard to calculate artemisinin in each of part of the mutant lines and parent plant.

RESULTS AND DISCUSSION

Study for increasing of artemisinin production using biotechnology including tissue culture, artemisinin biosynthesis regulation, genetic engineering and bioreactor have been presented by Liu *et al.* (2006), however, study of mutation induction by gamma rays irradiation for improving of artemisinin content in each part of plant is still limited in its publication.

The previous paper showed that roots of transformed *A.cina* by *Agrobacterium rhyzogenes* could produce 66.66 ppm artemisinin (Aryanti, 2001), the same phenomena was also been found at the experiment done by Xie *et al* (2001). According to Supaibulwatana *et al* (2004), it was found that artemisinin content increased from 6.15mg/g to 17.15mg/g in irradiated callus of *A.annua* at the doses of 50 Gy, furthermore, Koobkokkruad *et al* (2008) found that artemisinin content increased from 0.7% to 0.18% in shoot cultures of *A.annua* irradiated at the dose of 8 Gy. Roots of C27b1 mutant line was the highest artemisinin content compared to other lines, and no artemisinin was found in parent plant roots (Table I). Mutation induction at the doses of 10, 20 and 40 Gy improved artemisinin content in roots part of found in 2, 3 and 4 *A.cina* mutant lines, respectively.

Tabel II. Effect of gamma rays on morphological characters after two weeks of M1V3 *A.cina* in vitro cultures

Doses (Gy)	Mutant lines	Plant height (Cm)	Number of leaves/plantlet	Leave area (Cm^2)
0	Parent plant	6.0a	7a	0.8a
10	A6a1	5.0b	8a	1.3d
	A10.2	4,6c	10b	1.1c
	A11.3	5.0b	10b	0.6b
	A17.1	6.0a	11b	1.2c
	A17.2	6.5d	12bc	1.0c
	A19a1	5.8a	9b	1.2c
	A21.3	5.2b	10b	0.9a
	A26a3	5.4e	9ab	1.1c
20	B1a2	4.5c	7a	1.7ef
	B7.2	5b	7a	0.6b
	B10.2	4.8bc	10b	1c
	B12.1	4.5c	8a	1.2c
	B16.2	5.7ae	7a	1.8f
	B28.2	5b	9ab	0.9a
	B24a2	4.8bc	8a	1c
	B24b3	5.5ae	9ab	0.9a
40	C8.3	4.7bc	6d	2g
	C11.5	3.8f	5d	1.8f
	C11b2	4.8bc	6d	1.7ef
	C11b3	3.7f	8a	1.5e
	C15a2	4.8bc	5d	2e
	C18.2	4.3c	8a	1.7ef
	C27a1	4.3c	7a	2.3g
	C27b1	3.9f	8a	1.6ef

Note : Different letters in the same column indicating significant differences at the 5% level.

Although stems as traffic trunk of nutrients however they also contain bioactive compound, as proven by *Aglaia argentea* whose bark extract could inhibit Leukemia L_{1210} with IC_{50} of 0.06ppm (Aryanti, 1999). Related to bioactive compound, artemisinin was also found in stems of B12.1 and C8.3 mutant lines. According to Croteau and McCaskill (1998), artemisinin is formed through mevalonic-acetic with 3-hydroxy-3-methylglutaryl-coenzim A reductase (HMGR) regulator, this gene is more expressed in leaves and flowers compared to roots and seed. Abdin *et al.* (2003) have proven that the highest artemisinin content when the plants bloom or before flowering. Moreover, it was indicated that flowering was controlled by *fpf1*, a gene related to artemisinin biosynthesis. Associated with gene expression, artemisinin content was 36.68mg/g in flowers of A17.1 mutant line and 73.13mg/g in leaves of A26a3 mutant line. The same phenomenon was also occurred in C27b1 mutant line irradiated by 40 Gy, which has 36.94mg/g artemisinin in leaves. In very low artemisin content, however, has been observed in every part of un-irradiation plants. Irradiation dose of 10 Gy could improve artemisinin content in roots at 2 mutant lines, 1 mutant line at stems, 3 and 5 mutant lines in flowers and leaves respectively. Mutation induction at dose of 20 Gy indicated those 2 mutant lines increased the artemisinin content in roots and 1 mutant line in stems and leaves respectively. Irradiation dose of 40 Gy was the best dose compared to other doses, increasing artemisinin content in 4 mutant lines in roots part, 3 mutant lines in stems, and 1 and 6 mutant lines in flowers and leaves.

Table III. Dry weight of roots, stems, leaves and flowers per plant of *A.cina* mutant lines from 10, 20 and 40 Gy gamma rays doses

Doses (Gy)	Mutant lines	Roots (g)	Stems (g)	Flowers (g)	Leaves (g)
0	Parent plant	35,85a	30,88a	4,1a	13,55a
10	A6a1	38.42a	23.82de	4.24a	26.40e
	A10.2	27.75de	22.52d	7.52dc	16.90c
	A11.3	60.00i	35.57f	NF	38.81
	A17.1	46.84h	30.67a	7.21c	40.86g
	A17.2	28.93e	12.49bc	NF	12.31a
	A19a1	31.67f	20.09d	7.00c	16.82c
	A21.3	20.00c	22.19	7.14c	16.70c
	A26a3	25.42d	14.43c	NF	15.02a
20	B1a2	22.8dc	21.58d	4.77a	20.51d
	B7.2	25.02d	25.73e	NF	15.24a
	B10.2	28.14e	34.26f	NF	24.05e
	B12.1	15.5b	9.07b	NF	7.44b
	B16.2	37.07a	23.38d	NF	17.17c
	B28.2	28.26e	30.00a	2.54b	18.98d
	B24a2	31.78f	24.03e	12.64f	15.76ac
	B24b3	23.44dc	10.77b	6.96c	15.15a
40	C8.3	102.24m	48.13h	NF	41.65g
	C11.5	72.88k	39.50g	6.50c	38.40g
	C11b2	65.24j	42.18g	NF	39.88g
	C11b3	70.45k	26.62e	8.10d	29.48f
	C15a2	42.01g	27.85e	4.90a	49.61h
	C18.2	107.08n	25.82e	10.35e	23.92e
	C27a1	85.12l	26.02e	8.98d	48.07h
	C27b1	64.48j	20.02d	Tb	21.29d

Note : NF: No flower. Different letters in the same column indicating significant differences at the 5 % level.

Morphological variation

When *A.cina* buds were *in vitro* exposed by three doses of gamma rays, 95% of buds grow well as indicated by elongation of buds until sub culture. Sub culture was conducted after 3 weeks of irradiation, followed by selection of mutant lines based on morphological and performance of plants. Eight numbers of selected mutant lines dose are displayed in table II.

The variation of mutant lines from mutation induction at the doses of 10, 20 and 40 Gy toward plant height, number of leaves per plant and leaves area.

In general, all the mutagenic treatments caused a reduction of plant height compared to the parent. The maximum plant height which 6.5cm was found in A17.2 mutant line and the minimum plant height 3.7cm in C11b3 mutant line from mutation induction dose of 10 Gy and 40 Gy respectively. Number of leave per plantlet was also affected by gamma dose, the dose of 10 Gy improved the number of leave per plantlet compared to control plant at doses of 20 and 40 Gy. Low dose of gamma rays induced number of leaves but higher dose improved area of leave as indicated in mutant lines at the dose of 40 Gy. Charbaji and Nabulsi (1996) reported that the increasing of grapevine shoot length at the dose of 7 Gy and increasing of roots at the dose of 5 Gy. The number of leaves per plant were aslo increased by both doses. This character is similar with the irradiated *Amorphophallus muelleri* invitro culture,

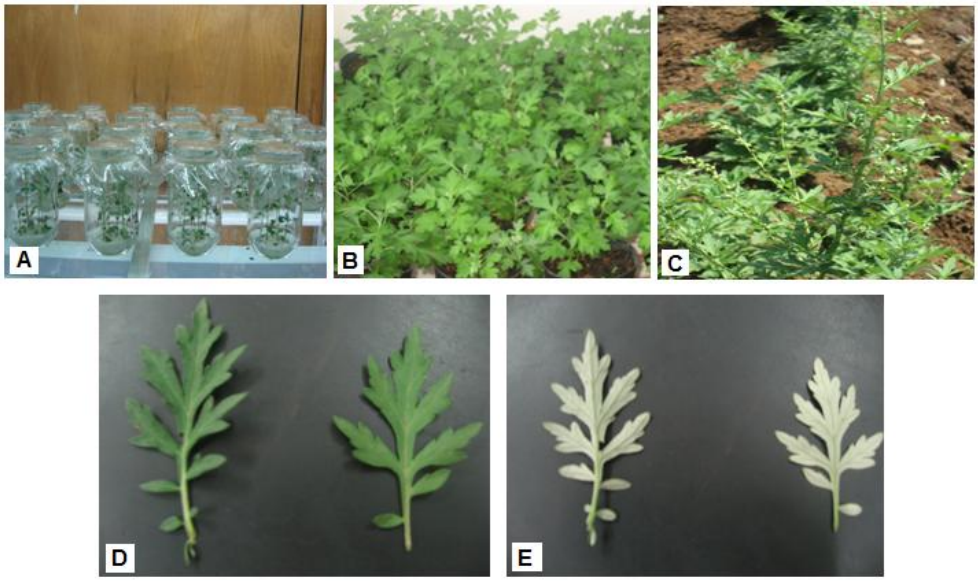

Figure 1. Tissue culture (A), acclimatization (B), field trial (C), leaves character (D and E) of *Artemisia cina* mutant lines

Table IV. Dry weight average of roots, stems, flowers and leaves at each dose of gamma rays

Doses (Gy)	Roots (g)	Stems (g)	Flowers (g)	Leaves (g)
0	35,85a	35,85a	4,10	13,55a
10	34,75a	22,88b	4,13	22,98b
20	26,50b	22,35b	4,38	16,79a
40	76,19c	32,02a	4,14	36,54c

Note : Different letters in the same column indicating significant differences at the 5 % level.

showed that increasing the dose up to 4 Gy improved the number of buds and multiplication rate (Poerba, *et al.,* 2009).

Mutation induction by gamma rays causing genetic and phenotype variation as dwarf, dense, broadleaf and reduce leaves area compared to un irradiated plant. At the previous paper it was presented that plant height of mutant line A25.2 was taller but smaller leave area than the parent plant. Performance of plants vary in field trials and also produce different dry weight of each plant part (Table III). Function of roots are absorbing nutrients from soil and causing plants to grow well. Stem is an important part of the plant to supply nutrients from the roots to rest of the plant. In comparison between doses of 10, 20 and 40 Gy on roots and stems performance of mutant lines, indicated that mutant lines from the dose of 40 Gy have more roots and bigger stems compared to other doses, this character is shown by dry weight of mutant lines in (Table II). The heaviest dry roots was reached by C18.2 mutant line, and the heaviest dry leaves is found in C15a2 mutant line. However, mutation at the dose of 20 Gy affected roots, stems and leaves of B12.1 mutant line. The best dose for improving plant growth in the field is at the dose of 40 Gy and 10 Gy.

Figure 1 display of plantlet in sterile Murashige & Skoog medium (A), plant after acclimatization in the green house (B), plants in field trial after 2 months cultivation (C), leave character of mutant lines (D and E).

CONCLUSSION

Physical mutagenic of gamma rays could induce artemisinin content. Artemisinin could be produced in all parts of *A.cina* mutant lines and the artemisinin content was increased by the dose of 10 and 40 Gy. The highest artemisinin content was found in leaves of A26a3 and C27b1 which were 73.13mg/g and 36.94mg/g, and at flowers of A17.1 which is 36.68mg/g respectively. Artemisinin content increased from 0.05mg/g in root parent plant to 1.90mg/g in roots of C27a1 mutant line, and 1.50mg/g artemisinin in stems of C8.3 mutant line. The dry weight of mutant lines leaves higher than that of parent plant by each dose, and improving dry weights of roots only by dose of 40 Gy. Dose of 40 Gy was the best dose for mutation induction for enhancing artemisinin content.

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