

# Leaf-variegated mutations induced using gamma irradiation of *Anubias minima*

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## ABSTRACT

The effects of chronic gamma irradiation on leaf mutations of *Anubias minima* were investigated. Tissue cultures of *A. minima* were exposed to chronic gamma radiation in a gamma room using Cobalt-60 sources at the Nuclear Technology Research Center, Faculty of Science, Kasetsart University, Bangkok, Thailand. Samples received a total dose of 0, 26.58, 41.12, 65.31, 79.81, 103.81, and 127.91 Gy at a dose rate of 0.6 Gy/h. At 60 days after irradiation, the number of surviving plantlets and the number of new shoots were counted and compared with the controls (0 Gy) to calculate the 50% lethal dose (LD<sub>50</sub>) and 50% growth reduction (GR<sub>50</sub>). The results showed that the radiation dose did not affect plant survival, but the GR<sub>50</sub> was about 125 Gy in the M<sub>1</sub>V<sub>1</sub> generation. After that, subcultures of new plantlets were transferred to a new medium, and the next generations were observed to note any new mutated characteristics after gamma irradiation. In the M<sub>1</sub>V<sub>0</sub> generation, variegated leaf mutants could be selected. There were four different types of mutant leaves: Dark green leaves with light green specks, dark green leaves with white specks, light green leaves with dark green veins and specks, and white leaves with dark green veins and specks. After 3 months, the selected mutants of *A. minima* tissue transplanted into water tanks were healthy and had 100% survival.

## 1. INTRODUCTION

*Anubias* is an aquatic plant that is important to Thailand's economy because it is beautiful and popular for use in aquariums, which has created demand in both domestic and export markets. *Anubias* spp. are monocotyledonous angiosperms in the family *Araceae*. The stem is an underwater rhizome. The leaves are ovate to lanceolate, with a dark green color. *Anubias* spp. grow well at temperatures of about 25°C at pH 6.5–7.4 [1]. It can be propagated by separating the shoots or dividing the rhizome and cultivating new shoots [2,3]. Thailand has been cultivating aquatic plants for export for 30 years. Some farms produce aquatic plants for domestic sales and export in many areas, such as Bangkok, Nakhon Pathom, Krabi, and Phuket. The volume and export value of aquatic plants in 2015 for Thailand was 9.7 million plants with worth 1.179 billion USD [4]. The top-five aquatic plants with high market demand and export value are *Anubias*, *Nymphaea*, *Echinodorus*, *Hygrophila*, and *Cabomba* [5]. It can be seen that *Anubias* is the number one in demand for aquatic

plants. In 2018, *Anubias* had an export value of up to 13 million baht [6].

*Anubias* are extremely easy plants to grow and can survive a wide range of water parameters. *Anubias minima* has long and slender leaves in contrast to *Anubias nana*, which has shorter and rounder leaves. Like other *Anubias* plants, they can grow in both aquariums and terrariums, either submerged or emerged [1]. Experts estimate that the Thai aquatic plant market will continue to expand steadily and that Thailand's competitiveness in the market will increase even more if there is promotion and support for the production of standard aquatic plants. Exotic beauty from plants native to this area is in demand in the market. This research aimed to combine gamma irradiation with tissue culture techniques to improve *Anubias* varieties by inducing mutation to create beautiful new morphological traits.

## 2. MATERIALS AND METHODS

### 2.1. Propagation of *A. minima* in Tissue Culture

*A. minima* plants were propagated in tissue culture on MS medium [7] with sugar 3% and gelrite 2.5 mg/L to create new shoots. When enough samples of *A. minima* were available for the experiment, the 8-week-old *A. minima* tissue was exposed to chronic gamma irradiation.

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## 2.2. Chronic Gamma Irradiation

Tissue cultures of *A. minima* were exposed to chronic gamma radiation in a gamma room using a Cobalt-60 source at the Nuclear Technology Research Center, Faculty of Science, Kasetsart University. Different treatment groups of samples received total doses of 0, 26.58, 41.12, 65.31, 79.81, 103.81, or 127.91 Gy with a dose rate of 0.6 Gy/h. After irradiation, the plantlets were subcultured to a new MS medium for the  $M_1V_1$  generation. At 60 days after irradiation, the number of surviving plantlets and the number of new shoots were recorded to calculate the 50% lethal dose ( $LD_{50}$ ) and 50% growth reduction ( $GR_{50}$ ) in comparison with the control (0 Gy).

## 2.3. Observing the Appearance of Leaf Mutations after Chronic Gamma Irradiation

After recording and calculating the initial data at the  $M_1V_1$  generation, the surviving plants were again subcultured into new tissue culture vessels of MS medium for the  $M_1V_2$  generation. They were observed for any morphological changes from the control, and new plantlets and new shoots from the  $M_1V_2$  generation with changed morphology were separated to form the  $M_1V_3$  generation. In the following generations, mutant plantlets were then screened and maintained separately from normal plantlets until it was found that the selected mutations were stable.

## 2.4. Chlorophyll Content Measurement on Leaf Mutants of *A. minima*

Chlorophyll content was determined in the leaves of mutated *A. minima* in the  $M_1V_{10}$  generation, based on an adapted method described by Hipkins and Baker [8].

## 2.5. Experimental Design and Statistical Analysis

The experiment design was a completely randomized design with three replications. The data were analyzed using analysis of variance, after which means were compared using the least significant difference. The analyses were facilitated by the R program [9].

## 3. RESULTS AND DISCUSSION

### 3.1. Effect of Chronic Gamma Irradiation on Survival and Growth Rate in Tissue Culture of *A. minima* in the $M_1V_1$ Generation

After irradiation, the results showed that chronic gamma irradiation did not affect *A. minima* tissue survival. When data were statistically

analyzed, there was no significant statistical difference [Table 1]. The  $LD_{50}$  could not be calculated because the irradiated sample had a high survival percentage of more than 50% [Figure 1]. This is similar to the experiments of Tangpong *et al.*, Jompuk *et al.*, and Sukin *et al.* [10-12], which reported that chronic gamma irradiation had no effect on the survival rate in the tissue culture of *Anubias congenensis* N.E. Brown, *Cryptocoryne wendtii* "brown," and *A. nana*.

Our study of the effect of chronic gamma irradiation on growth rate found that when the radiation dose increased, the growth rate percentage decreased. At the maximum radiation dose (127.91 Gy), the lowest growth rate was 48.45%. The statistical analysis showed that the irradiated samples had a significantly lower growth rate than the control [Table 1]. Therefore, the  $GR_{50}$  could be calculated as 125 Gy [Figure 1].

### 3.2. The Selected Leaf Mutants of *A. minima* after Chronic Gamma Irradiation

After irradiation, the survival and growth rate data were collected at the  $M_1V_1$  generation. After that, the new plantlets were put into new bottles of MS medium and called the  $M_1V_2$  generation. The morphological changes were observed in the new plantlets, and putative mutants were separated from the normal-looking plantlets. The results showed a variety of mutations, but the leaf mutation was the most apparent example. The mutants were screened up to the  $M_1V_{10}$  generation. Four types of stable leaf mutations were found: Dark green leaves with light green specks, dark green leaves with white specks, light green leaves with dark green veins and specks, and white leaves with dark green veins and specks [Figure 2]. The most frequent leaf mutations were found at the dose of 79.81 Gy. This was similar to the findings of Tangpong *et al.* [10], which found that after chronic gamma irradiation, the most common mutation observed in the  $M_1V_3$  generation of *A. congenensis* was albinism. In the reports of chronic gamma irradiation on *A. nana*, the mutant characteristics in the  $M_1V_4$  generation were variegated leaves, dwarfism, albinism, light green leaves, and abnormal leaves [12] and after chronic gamma irradiation, variation observed in the  $M_1V_2$  generation of *C. wendtii* "brown" were small narrow leaves, green leaves, paler brown leaves, smaller leaves, dwarfism, and increased branching [11]. In the Kim *et al.* experiment, which observed changes in both *Cymbidium* hybrids' leaf color and shape after gamma irradiation, the leaf color mutants showed the highest mutation frequency and spectrum [13].

**Table 1:** Survival percentage and growth rate percentage of *Anubias minima* in the  $M_1V_1$  generation at 60 days after chronic gamma irradiation.

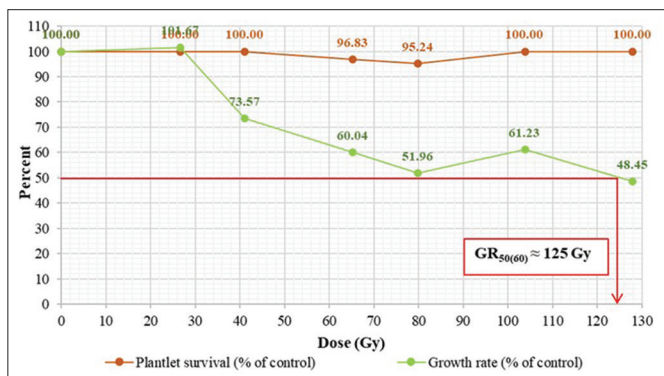
Radiation dose (Gy)	Total number irradiated	Number of surviving plantlets	Survival percentage (% of control)	Number of new plantlets	Growth rate percentage (% of control)
0	63	63	100.00	163	100.00 <sup>nl</sup>
26.58	45	45	100.00	118	101.67 <sup>a</sup>
41.12	54	54	100.00	102	73.57 <sup>b</sup>
65.31	64	62	96.83	97	60.04 <sup>c</sup>
79.81	63	60	95.24	84	51.96 <sup>cd</sup>
103.81	48	48	100.00	76	61.23 <sup>c</sup>
127.91	51	51	100.00	63	48.45 <sup>d</sup>
F-test			ns		**
LSD <sub>0.05</sub>			5.08		9.44
%CV			3.00		7.60

Ns: Non-significant at 5% level. \*\*Significant at 1% level. <sup>l</sup>means within columns followed by common superscript letters are not significantly different ( $P < 0.05$ )

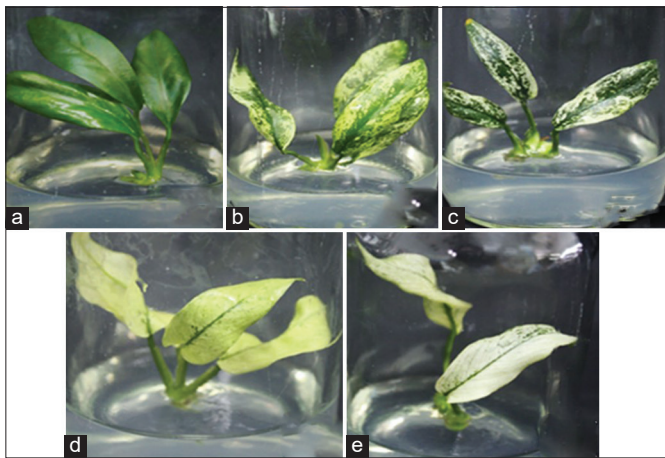
**Table 2:** Chlorophyll content of leaf mutants of *Anubias minima* in  $M_1V_{10}$  generation.

Type of leaf mutant	Chlorophyll a (mg/g of fresh leaf weight)	Chlorophyll b (mg/g of fresh leaf weight)	Total chlorophyll (mg/g of fresh leaf weight)
Control	2.308 <sup>a</sup>	1.179 <sup>a</sup>	3.520 <sup>al</sup>
Dark green leaves with light green specks	1.727 <sup>b</sup>	0.797 <sup>b</sup>	2.548 <sup>b</sup>
Dark green leaves with white specks	1.224 <sup>c</sup>	0.620 <sup>c</sup>	1.861 <sup>c</sup>
Light green leaves with dark green veins and specks	0.974 <sup>d</sup>	0.502 <sup>cd</sup>	1.490 <sup>d</sup>
White leaves with dark green veins and specks	0.753 <sup>e</sup>	0.381 <sup>d</sup>	1.145 <sup>e</sup>
F-test	**	**	**
LSD 0.05	0.206	0.144	0.069
%CV	8.12	11.43	1.79

\*\*Significant at 1% level. <sup>a</sup>means within columns followed by common superscript letters are not significantly different ( $P < 0.05$ )



**Figure 1:** Effects of radiation dose on survival percentage and growth rate percentage of *Anubias minima* in the  $M_1V_1$  generation at 60 days after chronic irradiation.



**Figure 2:** Different types of leaf mutations in the  $M_1V_{10}$  generation after irradiation: (a) Control (non-irradiation), (b) dark green leaves with light green specks, (c) dark green leaves with white specks, (d) light green leaves with dark green veins and specks, (e) white leaves with dark green veins and specks.

### 3.3. Chlorophyll Content Measurement on Leaf Mutants of *A. minima*

Fresh leaves from mutant *A. minima* leaves were used to determine the amount of chlorophyll content, and absorbance was measured using spectroscopy at 650 and 665 nm wavelengths. The measured values were used to calculate the content of chlorophyll A, chlorophyll B, and total chlorophyll.



**Figure 3:** The color of the solution extracted from leaf mutants of *Anubias minima* in the  $M_1V_{10}$  generation: (a) Control, (b) dark green leaves with light green specks, (c) dark green leaves with white specks, (d) light green leaves with dark green veins and specks, (e) white leaves with dark green veins and specks.

The results showed that the chlorophyll content of four types of mutated leaves was different and lower than the control and decreased when the leaves were lighter in color. When statistically analyzed, there was a statistically significant difference. The leaves with the highest chlorophyll contents in the mutant leaf samples were dark green with light green specks, followed by dark green with white specks, light green with dark green veins and specks, and white with dark green veins and specks, respectively [Table 2]. Chlorophyll content can be used to estimate that the color of the leaves will change if the chlorophyll content varies. For example, mutant leaves with more dark green leaf surfaces than those with light green and white leaf surfaces had higher chlorophyll contents, which was the darker color of the solution [Figure 3]. In concordant research on gamma irradiation in *Plantago ovata* [14] and *Curcuma* hybrid “Laddawan,” it was found that gamma irradiation affected chlorophyll content, with irradiated samples having less chlorophyll content than the control (non-irradiated) [15]. In addition, Chanu *et al.* studied in tree tomato with gamma irradiation at doses of 0, 10, 25, 50, 75, and 100 Gy and reported that chlorophyll content decreases with the increase in gamma dose, and irradiated samples had less chlorophyll content than the control [16]. Yamaguchi, *et al.*

reported that exposure to carbon ion beam, helium ion beam, and gamma rays induced albina, xantha, viridis, and other mutants such as striata (longitudinal white or yellow stripes) and maculate (green or yellow spots distributed over the leaf) [17].

#### 4. CONCLUSION

The chronic gamma irradiation by cobalt-60 source can make leaf mutants of *A. minima*. The selected mutant plants can be transplanted with healthy and survive in water tanks.

#### 5. ACKNOWLEDGMENTS

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#### 6. AUTHOR'S CONTRIBUTIONS

This work is part of the B.Sc. project of Mayuree Limtiyayotin (ML) advised by Peeranuch Jompuk (PJ). ML and PJ have designed the study, conducted the experimental work, and corrected the data. Choosak Jompuk (CJ) contributed to the data and statistical analysis. ML wrote and PJ and CJ revised the manuscript. Natnichaphu Sukin is the scientist who takes care of the instruments and tissue culture laboratory.

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#### 8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

#### 9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

#### 10. DATA AVAILABILITY

The data that support the findings of this study are available on request from the corresponding author.

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