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Effects of γCo-60 radiation on the growth and development of peppermint (*Mentha arvensis* L.)

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Abstract: In this study, mints were regenerated from calluses irradiated by γ - rays (Co-60) at doses from 0 to 70 Gy. After 4 times of propagation, the M_1V_4 mints were transferred in a greenhouse for assessments of growth, development and essential oil content. Mint regenerated from calluses irradiated at low doses (0, 3, 4, 6, 8 and 10 Gy) showed no morphological difference compared to the control plants. However, low-dose irradiated mints showed better effect on growth and development, and mints regenerated from 6Gy-irradiated sample had essential oil content with 1.3 times higher than that of control group. In case of plants irradiated at higher dose (30, 40, 50, 60 and 70 Gy), there were morphological variations such as stem become bigger and color of stem become purple. Especially, samples irradiated at 60 Gy showed better in growth, development and essential oil content (1.34 times higher than the control plants) and these characters were stable in M_1V_4. Among the irradiated mints, purple stem variations had the highest essential oil yield with 1.7 times higher than that of the original plants.

Keyword: *mint, γ - radiation, Mentha arvensis L., variation, essential oil.*

I. INTRODUCTION

Nowadays, the applications of mutation breeding are getting more and more encouraging outcomes worldwide. Back in 1960, there was only 6 mutagenic plants; in 1968: 50; 1970: 80; 1980: 500; 1982: 900; 1988: 1200; and in 2000 the number of mutagenic plants reached 2255 [1]. By 2010, over 3100 mutagenic plant varietals have been generated by radiation treatment [2]. These mutagenic plants have higher yield, better quality, shorter growing season, more adaptable to salty water, acid soil and drainage, better resistant to insects and infections, smaller in size which would reduce the farming area and easier to harvest, etc. [3,4]. In particular, irradiation combined with *in vitro* cultivation was first studied in the 1990 on bananas and potatoes. By today, this method has been used on many plants and various studies have already been conducted on medical plants in order to boost the yield of the secondary metabolites.

Currently, peppermint oil is widely used in many industrial sectors, especially Asian Peppermint oil because of its high levels of menthol. Menthol is now being largely consumed in India with the estimated consumption about 10,000-15,000 tons. Moreover, the estimated consumption of menthol in China is 3,500 tons and 1,900 tons in Europe which consequently brings the global consumption to 18,200 tons [5,6,7]. Although essential oil is being considered as white gold of the food industry, how to reduce the price of oil in general and the price of menthol in particular in order to cut down the

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use of chemically synthesized essential oil and menthol in food as well as cosmetic products for consumer health protection is still an unanswered question [8]. Under these circumstances, it is necessary to created mutational mint using γ - (Co-60) radiation and screening for peppermint (*Mentha arvensis* L.) varietals which are well adapted with natural environment, have good resistance to insects and infection, greater yield and higher concentration of menthol in essential oil in order to satisfy the industrial demands as well as the consumers' habit. Besides that, irradiated mint would probably provide us with a lot of potential applications in medical plants studying, especially in enhancing the concentration of secondary metabolites.

II. MATERIALS AND METHODS

Materials

Callus samples were regenerated from peppermint namely *Mentha arvensis* L. Urea and NPK fertilizers were used for trial cultivation in greenhouse in combination of COC 85 and Kasumin fungicides treatment. Gamma cell used for irradiation was a gamma Co-60 source from India (BRIT 5000) at Dalat Nuclear Research Institute. Essential oil in samples was extracted by a steam distillation method while the menthol content was evaluated by gas chromatography.

Preparation of irradiated peppermint for evaluation of growth and development

3-weeks calluses samples were irradiated in 500ml-glass bottles at low doses (3, 4, 6, 8 and 10 Gy) and high doses (30, 40, 50, 60 and 70 Gy). Irradiated samples were then transfered to new bottles containing new medium for regeneration of shoot buds. The shoots buds grown from irradiated callus were cut and used for shoot multiplication until M1V4 before culturing in rooting media for plantlets formation. In case of samples irradiated at high dose, the variation individuals were screened and separated from shoot buds generated from irradiated calluses in M1V1. All type of variation such as bigger stem, purple stem and so on were selected and cultured until M1V4 generation for evaluation the stability of variant character.

To evaluate the growth and development of irradiated peppermint and variation individuals, the plantlets regenerated in M1V4 were transfer for plantation in a greenhouse and the shoot height, survival rate, branch number and oil content were determined at 20, 40 and 60 days. All plantlets generated from each irradiation dose were planted in the same bed with completely randomized design (CRD). All experiments were repeated three times. Data were statistically analyzed by variance analysis (ANOVA) and means were compared using the least significant difference (LSD) at 5% probability level and standard deviation Duncan's multiple range test [9].

Determination of oil and water contents in peppermint

Mint after cultivated on field was harvested and extracted for collecting oil using steam distillation method when the flowering rate reached 30%. Water content was also being determined at the same time while the essential oil was being extracted.

Evaluation of changes in the terpenoid content of peppermint oil

Evaluation of maximum absorption peaks: Standard menthol diluted with ethanol was subjected into OD measurement on an UV-vis spectroscopy for maximum absorption peak examination.

Terpenoid content in peppermint oil: Samples were diluted by pure ethanol by 1000 folds (5 µl of essential oil in 5 ml of pure ethanol), absorbance peaks were measured by an UV-vis spectroscopy at 203 nm of wavelength.

Determination of menthol by gas chromatography

After the terpenoid concentration in oil was evaluated by measuring the absorbance using UV-vis, the most terpenoid-containing oil samples (60 Gy irradiated – purple stems mint line 2) and original mint oil samples were subjected into menthol evaluation using gas chromatography (extracted oil was diluted 2500 folds with dichloromethane). The main goal of this experiment is to determine menthol concentration is essential oil. Afterward, the menthol content was analyzed by gas chromatography machine (Agilent Technologies 6890 N Network GC System), FID probes (flame ionization detector), ZB-5-MS 30 mm x 0.25 mm x 0.25 μm silica coated column. Temperature profile was from 90 to 180^0C, 3^0C/minute. Injection port and probes temperature was 220 and 230^0C. N_2 flow rate: 1 ml/min [10].

III. RESULTS AND DISCUSSION

Evaluation of growth and development of peppermint

Evaluate the growth of peppermint irradiated at low dose (0-10 Gy): It can be seen from table I that after 20 days of cultivation, the plants height calculated from these experiments varied slightly from 8.1 to 12.2 cm. Peppermints irradiated with γ -radiation at the dose of 4 and 8 Gy had average heights of 12.2 and 12.6 cm, which was not significantly different compared to the original plants (10.3 cm). However, peppermints treated with 4 and 8 Gy irradiation showed statistically different in height compared to the original ones. After 40 days of cultivation, the mint's height varied between 20.8 and 27.6 cm (Fig.1). The heights of 6 and 8 Gy irradiated mints were 27.1 and 27.6 cm, which were significantly different to compare with the original (20.8 cm) and other irradiated plants. Furthermore, after 60 days of cultivation, mint's height varied from 26.7 to 31.4 cm. The treated mints at 6 and 8 Gy were attained the greatest height by 31.3 and 31.2 cm to compare with those of other samples. However, the height was showed no statistical difference to compared to the original plants. The development and growth of peppermint indicated that at irradiation dose of 6 and 8 Gy, the height growth was enhanced the best compared to the original and other irradiated plants, which led to a conclusion that γ - radiation had some certain effects on mint's height development.

Table I. The plant height low-dose irradiated mints

Dose (Gy)	Plant height (cm)		
	20 days	40 days	60 days
0	$10.3^{ab} \pm 2.9$	$20.8^{a} \pm 4.3$	26.7 ± 4.3
3	$9.8^{ab} \pm 0.9$	$25.3^{ab} \pm 1.6$	29.9 ± 1.2
4	$12.2^{b} \pm 1.8$	$23.9^{ab} \pm 0.9$	26.9 ± 0.9
6	$8.7^{a} \pm 0.9$	$27.1^{b} \pm 1.6$	31.4 ± 1.3
8	$12.6^{b} \pm 0.4$	$27.6^{b} \pm 2.7$	31.2 ± 2.4
10	$8.1^{a} \pm 1.6$	$24.3^{ab} \pm 6.0$	29.2 ± 7.6

In the same column, means followed by the same letter are not significant different at the 0.05 level of probability

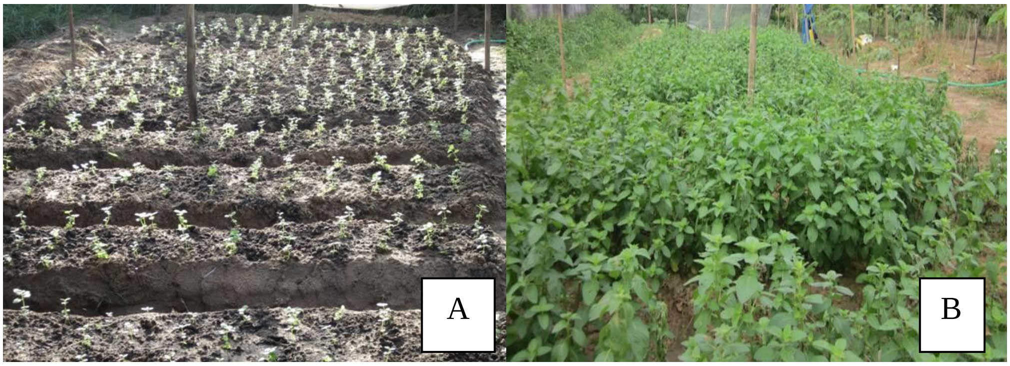

Fig. 1. Field cultivation of mint: after 1 day (A) and 40 days (B) of cultivation.

Table II. Branches development of samples irradiated at low doses

Dose (Gy)	Number of branches (branches/plant)		
	20 days	40 days	60 days
0 (control)	$2.7^{ab} \pm 2.9$	$6.8^{a} \pm 3.7$	$9.9^{a} \pm 4.8$
3	$3.3^{ab} \pm 0.7$	$7.2^{a} \pm 1.3$	$10.2^{a} \pm 1.6$
4	$4.2^{ab} \pm 2.9$	$13.2^{b} \pm 2.5$	$17.3^{b} \pm 3.6$
6	$3.2^{ab} \pm 1.0$	$12.8^{b} \pm 2.4$	$17.2^{b} \pm 18.9$
8	$5.6^{b} \pm 1.0$	$13.1^{b} \pm 2.1$	$17.5^{b} \pm 3.4$
10	$1.7^{a} \pm 1.7$	$10.7^{ab} \pm 0.9$	$16.7^{b} \pm 0.6$

According to the statistical tests and $LSD_{0.05}$ classification, the branches development between the tested plants were statistically different at 0.05 level of probability (see table II). After 20 days of cultivation, the branches number of sample irradiated at 8 Gy was highest with 5.6 branches/plant on average compared to those of the original and samples irradiated at other doses. After 40 days of cultivation, plants irradiated at the dose of 4 and 8 Gy had the most branches (which were 13.2 and 13.1 braches/plant on average) compared to the non-irradiated (6.8 braches/plant on average) and other-dose treated ones. The outputs have reconfirmed that γ-(Co-60) radiation caused some certain impacts on the branching speed of peppermints.

In addition, the irradiated mints were also monitored for the survival rate until harvested. The results shown in table III indicated that the samples irradiated by 8 Gy revealed more resistance to ecological conditions and infections than those of the other samples, leading to the survival rate was the highest by 81.3%. However, the sample irradiated 6 Gy had the best results in growth and development as well as highest terpenoid concentration compared to the others. Therefore, when all of the relative factors were combined, 6Gy-irradiated plants showed the best results in growth, development and essential oil content.

Evaluate the growth of peppermint irradiated with high dose: As showed in Fig. 2, γ-rays radiation had certain impacts on peppermint's height throughout its growth and development stages. The results from table IV indicated that after 20 days of cultivation, there was a statistical difference between the plant heights of tested samples. Mint height between the experiments varied widely from 8.8 to 15.6 cm. In addition, 60Gy-irradiated-purple stem mints line 2 had the highest shoot height with 15.6 cm, and next was the shoot height of 60Gy-irradiated-purple mints line 3 with 15.3 cm (this value of the control one was only 10.3 cm). After 40 days of cultivation, mint height varied between 23.9 and 29.2 cm, respectively. At this stage, the shoot heights of 60Gy-irradiated mints (includes 60Gy-irradiated-purple stems variation line 1, 2 and 3) were much higher than those of other plants. At the stage of 60 days of cultivation, 60Gy-irradiated plant again achieved the highest shoot height compared to those of other irradiated and original plants. These differences in shoot height indicated that beside the capability of morphological mutagenesis, γ-rays radiation at certain doses also stimulated and enhanced mint growth.

Table III. Survival rate of samples irradiated at low doses

Dose (Gy)	Number of plant	Number of death	Number of survived plant	Survival rate (%)
0 (control)	66	20	46	69.7^{a}
3	60	15	45	75.0^{a}
4	48	15	33	68.8^{a}
6	51	17	34	66.7^{a}
8	48	9	39	81.3^{b}
10	57	18	39	68.4^{a}

Table IV. The plants height of samples irradiated at high doses

Dose (Gy)	Plant height (cm)		
	20 days	40 days	60 days
0 (control)	$10.3^{ab} \pm 2.9$	$21.1^{a} \pm 4.3$	$26.7^{a} \pm 4.3$
30	$8.8^{a} \pm 0.8$	$26.5^{ab} \pm 3.2$	$30.3^{ab} \pm 3.1$
40	$11.4^{ab} \pm 3.1$	$27.2^{ab} \pm 3.5$	$30.8^{ab} \pm 3.7$
50	$10.4^{ab} \pm 3.1$	$26.1^{ab} \pm 5.2$	$30.2^{ab} \pm 4.4$
60	$13.7^{bc} \pm 1.9$	$29.1^{b} \pm 5.1$	$32.2^{ab} \pm 4.6$
60M	$13.5^{bc} \pm 0.9$	$25.8^{ab} \pm 0.7$	$32.3^{ab} \pm 1.8$
60T1	$11.2^{ab} \pm 0.8$	$29.2^{b} \pm 0.3$	$33.5^{b} \pm 0.7$
60T2	$15.6^{c} \pm 0.9$	$28.0^{b} \pm 3.5$	$32.4^{ab} \pm 3.8$
60T3	$15.3^{c} \pm 0.4$	$27.7^{ab} \pm 2.6$	$32.4^{ab} \pm 3.3$
70	$10.6^{ab} \pm 0.6$	$23.9^{ab} \pm 4$	$27.0^{ab} \pm 3.0$

60 M: 60Gy-irradiated mint, bigger stem variation; 60 T1: 60Gy- irradiated mint, purple stem variation line 1; 60 T2: 60Gy-irradiated mint, purple stem variation line 2 and 60 T3: 60Gy-irradiated mint, purple stems variation line 3.

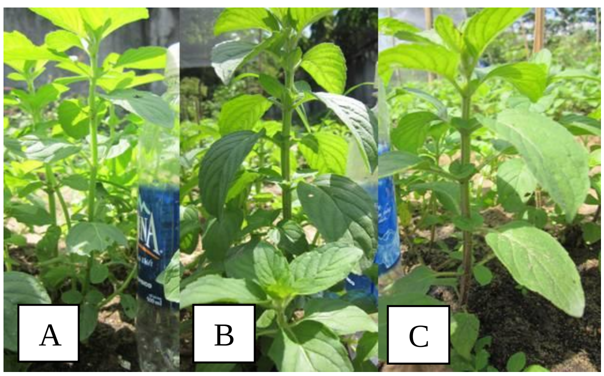

Fig.2. Photos of variation mints in the field, A: Control mint, B: bigger stem mint, and C: Bigger and purple stem mint.

Table V indicated that after 20 days of cultivation, there was no significant difference from branches development between the cultivated plants. However, after 40 days of cultivation, the branches number between the treated plants was statistically different. 40 Gy, 60 Gy and 60Gy-irradiated purple stem mint had higher branches which was 12.8, 15.1 and 11.0 branches/plant compared to other irradiated plants. After 60 days of cultivation, 60Gy- irradiated purple stem mint line 3 had the highest branches number at 18 branches/plant, which led to a conclusion that γ-rays radiation may affect to the growth and development of irradiated peppermint.

Table V. Branches development of samples irradiated at high doses

Dose (Gy)	Number of branches (branches/plant)		
	20 days	40 days	60 days
0 (control)	2.7 ± 2.9	$6.8^{a} \pm 3.7$	$9.9^{a} \pm 4.8$
30	3.1 ± 0.2	$7.8^{a} \pm 0.4$	$13.2^{ab} \pm 1.8$
40	4.4 ± 2.8	$12.8^{ab} \pm 5.4$	$17.6^{ab} \pm 5.2$
50	3.2 ± 2.6	$9.9^{ab} \pm 1.1$	$14.9^{ab} \pm 2.7$
60	4.8 ± 3.1	$15.1^{b} \pm 3.9$	$20.4^{b} \pm 3.4$
60 M	2.2 ± 1.5	$7.4^{a} \pm 1.1$	$14.8^{ab} \pm 3.0$
60 T1	3.6 ± 1.1	$11^{ab} \pm 2.6$	$17.8^{ab} \pm 2.8$
60 T2	2.8 ± 1.0	$9.8^{ab} \pm 2.2$	$16.8^{ab} \pm 2.6$
60 T3	4.1 ± 1.8	$9.9^{ab} \pm 2.3$	$18^{b} \pm 4.7$
70	3.5 ± 4.3	$11.7^{ab} \pm 7.6$	$17.3^{ab} \pm 7.7$

Planted mint was screened for morphological and disease deaths until harvest to determine the survival rate. According to the results indicated in table VI, the high survival rates in greenhouse were found at the treatment with 30 and 40 Gy, which was 92.2 and 97.6%, while this value of the control one was 66.6%. The reason may due to γ-cause some changes in high-dose irradiated mints lead to better growth and resistance to diseases in greenhouse condition. Therefore, we concluded that 30 and 40 Gy of radiation dose could probably boost peppermint resistance to cultivation conditions.

Table VI. Survival rate of samples irradiated at high doses

Dose (Gy)	Number of plant	Number of death	Number of survived plant	Survival rate (%)
0 (Control)	66	20	46	69.9
30	42	1	41	97.6
40	51	4	47	92.2
50	46	10	36	78.3
60	48	19	29	60.4
60 M	54	19	35	72.2
60 T1	60	14	46	80.0
60 T2	42	14	28	81.0
60 T3	51	17	34	74.5
70	60	34	26	43.3

Determination of oil and water contents in peppermint

After 60 days of cultivation, when the flowering rate was around 30%, leaves were harvested for oil extracted. The average essential oil content of the control plants was found about 2.9 g/100g dried leaves. The result in Table VII also showed that mints irradiated at 3 to 10 Gy had oil content similar to the control plants. Even so, the oil content of mint irradiated at 6 Gy dose was higher 27.5% than that of the control one. Furthermore, 6Gy-irradiated mint also showed better in growth and development than other low-dose irradiated plants which consequently indicated that low-dose irradiation from 3 to 10 Gy had some effects on mint growth and development except oil content changes. On the other hand, the water contents in irradiated plants showed no difference to the control plants, which led to a conclusion that γ - radiation at low dose did not influence the water concentration in peppermint.

Table VII. Water and oil contents of mints irradiated at low-dose

Dose (Gy)	Oil content (g/100g of dried samples)	Water content (%)
0 (Control)	2.9	79.2
3	3.4	77.5
4	2.7	76.7
6	3.7	74.2
8	2.5	76.7
10	2.1	75.8

According to data in table VIII, mint exposed to γ-rays radiation from 30 to 70 Gy showed no significant difference in oil content compared to the control ones while 60Gy-irradiated mint with purple stems variations line 1 and 2 statistically differed. In addition, oil concentration in 60Gy-irradiated purple stems variation line 1 was 68.97% greater and 60Gy-irradiated purple stem variation line 2 was 72.4% higher than the control mint. Besides that, 60Gy-irradiated purple stem variation line 1 and 2 also showed a better in growth and development.

Table VIII. Water and oil contents of mints irradiated at higher dose

Dose (Gy)	Oil content (g/100g dried sample)	Water content (%)
0 (Control)	2.9^{ab}	79.2
30	2.4^{a}	73.3
40	2.1^{a}	74.2
50	2.1^{a}	75.0
60	3.9^{ab}	78.3
60 M	2.2^{a}	74.2
60 T1	4.9^{c}	76.7
60 T2	5.0^{c}	75.8
60 T3	4.5^{bc}	72.5
70Gy	3.3^{abc}	75.8

Thus, beside enhanced growth and development of peppermint, γ - (Co-60) radiation also enhanced their oil concentration. Moreover, mints irradiated at 60 Gy also showed morphological differences which boosted their growth, development and also increased their oil content compared to the non-irradiated samples, especially in the purple stems variations. In our standpoint, irradiation with γ-rays (Co-60) would be the main method that should be used to created peppermint variations with higher oil concentration and original mint enhancement.

Evaluation of changes in the terpenoid content of peppermint oil

The maximum absorption peak of standard menthol after diluted 1000 times by ethanol was screened by a UV-vis spectroscopy and the maximum absorption peak of terpenoid compounds, which mainly are menthol and menthone in the standard product was found at 203 nm (data not shown). The Terpenoid content in essential oil was evaluated via the value of OD at 203 nm. The results in table IX showed that terpenoid contents in essential oil extracted from all samples were almost no significant difference. Since the result in table VIII pointed out the oil contents of in 60Gy-irradiated purple stem mint lines 1 and 2 were much higher than those of control and other irradiated plants, the total terpenoid content in 100g dried leaves collected from 60Gy-irradiated purple stem mint lines 1 and 2 higher than those of control and other irradiated plants.

Table IX. OD results of peppermint oil

Dose (Gy)	OD_{203}
0 (Control)	1.9
3	2.0
4	2.2
6	2.1
8	1.5
10	2.0
30 T1	1.9
40 T2	2.1
50 T3	2.1
60	1.5
60 M	1.8
60 T1	2.2
60 T2	2.3
60 T3	1.4
70	1.6

Table XI. The menthol concentration of 60Gy-irradiated purple stem mints line 2

Dose (Gy)	Menthol content in oil (%)	Menthol content in dried leave (g/100g air dried ground)
0 (Control)	80.2	2.33
60Gy-irradiated purple stem mints line 2	81.1	4.06

Determination of menthol by gas chromatography

Menthol is the most important terpenoid of essential oil, therefore the content of this terpenoid in essential oil extracted from 60Gy-irradiated purple stem mint line 2 was analyzed by gas chromatography in order to compare with that of the control one. The results in table 10 indicated that the menthol content in essential oil extracted from purple stems mint line 2 was 81.1 % and showed negligibly significant difference to that of original plants (80.2 %). While, the total menthol content in 100 g air dried ground harvested from 60Gy-irradiated purple stem mints line 2 was increased 74% compared to that of the control one. Thus, the purple stems irradiated mint line 2 not only showed a higher in the yield of essential oil but also has a better oil quality oil indicated by its menthol concentration.

III. CONCLUSIONS

According to the results above, we concluded that the dose of 6 Gy enhanced the growth and development mint indicated not only by the gain of 17.6% in shoot height, 73.7% in number of branches but also by the gain of 27.6% in oil yield and 8.9% in terpenoid concentration compared to that of the untreated control.

Three morphological variation lines including 2 lines of purple stem and 1 line with bigger stems were screened in M_1V_4. The purple stem variation lines showed a better growth and development as well as a higher yield of extracted oil (in a comparison with the control, the oil concentration on variation purple stem mints line 1 and line 2 were increased in 68.96 and 72.4%, respectively. In addition, the analytical result by gas chromatography also proved that the quality of oil extracted from variation mint (60Gy-irradiated purple stem line 2) was much better than that of the original one.

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