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Original research article

Alteration of Leaf Anatomy of Handeuleum (*Graptophyllum pictum* L. Griff) due to Gamma Irradiation

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ABSTRACT

The leaves of the plant handeuleum (*Graptophyllum pictum* L. Griff) have long been used for traditional medicine in several regions in Indonesia. This study was aimed to determine the effect of gamma irradiation rate on the anatomy and phytochemical content of the leaf. The rates of gamma rays used were 0, 15, 30, 45, 60, 75, 90, and 105 Gy. Our results showed that gamma ray irradiation rate of 30 Gy produced leaves that contain anthocyanins and carotenoids, with the highest number of stomata and stomatal density compared with control plants. Stomatal index was found highest in the leaves with 45 Gy of gamma irradiation. High-rate gamma ray irradiation produced rigid, thick, and frangible leaves. A high rate of gamma irradiation, i.e. 75, 90, and 105 Gy, produces bigger palisade, sponges, and upper epidermis than the control plants, respectively. Our results showed an association between increasing rate of irradiation with alterations in the structure of leaf anatomy and phytochemical content of handeuleum.

1. Introduction

Handeuleum (*Graptophyllum pictum* L. Griff.) is a medicinal plant that has potential to be one of the flagship medicinal plants in Indonesia. Handeuleum leaf has long been used as a traditional medicine to cure various diseases. Manoi (2010) showed that the handeuleum of Bogor accession has a high phytochemical content that is useful for treatment. Induction of mutations can be applied to increase the high phytochemical content that may be used for the development of Indonesian traditional medicine. Gamma ray irradiation could affect the growth and development of plants by altering the physiology, biochemistry, genetics, and morphological changes in cells and tissues. A low rate of gamma rays could stimulate growth (Melki and Marouni 2010), increase chlorophyll content (Jan et al. 2013), and increase the content of carotenoids (Kim et al. 2005), whereas high rates of gamma rays could interfere with the activity of the enzyme (Stajner et al. 2009). This study was aimed to determine the effect of gamma irradiation rates on the anatomy and phytochemical content of handeuleum leaves.

2. Materials and Methods

Rates of gamma irradiation consisted of eight levels: 0, 15, 30, 45, 60, 75, 90, and 105 Gy. Each treatment consisted of 10 replicates, using the cuttings for irradiated materials. Irradiation was performed at Pusat Aplikasi Isotop dan Radiasi (PAIR; Center for Isotope and Radiation Applications) BATAN, Jakarta. Observation of leaf anatomical characteristics was conducted in the Microtechnique Laboratory of the Department of Agronomy and Horticulture, Bogor Agricultural University.

Plants were taken from the propagation of handeuleum cuttings of Bogor accession that has high phytochemical content. Cuttings with the length of three sections planted in polybags with soil as a medium:compost in the ratio of 2:1 (v/v). Cuttings of 1 month old that have rooted and produced new leaves with a height of approximately 15 cm were removed from the media, cleaned of soil, and its roots were wrapped in an aluminum foil. Cuttings were then irradiated using gamma rays from ionizing cobalt 60 using a gamma irradiator chamber 4000A (Irpasena, India). Cuttings that have been irradiated were immediately planted in new media, grown under small lemur for around 2 months, then removed to a polybag with a diameter of 15 cm, and maintained on the field with 55% paranet shade.

The observation of leaf anatomical characteristics was performed on 5-month and 2-week-old plants (18 weeks after irradiation) on

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transversal and paradermal slices using intact preparation method of fresh ingredients, including (1) the number of epidermal cells and stomata; (2) stomatal density obtained from the following calculation: stomatal density = Σ stomata/area of field of view (mm^2); (3) stomatal index obtained from the following calculation: stomatal index = [Σ stomata/(Σ stomata + Σ cell epidermis)] × 100; and (4) thickness of leaves, cuticle, and upper epidermis, length of palisade, thickness of sponges, and lower epidermis. Observations were made with a molecular digital microscope.

Analysis of the total content of pigments such as chlorophyll, total carotenoids, and anthocyanins was conducted with the method by Sims and Gamon (2002), using a spectrophotometer.

3. Results

3.1. The content of anthocyanin, total chlorophyll, and carotenoids

Based on the results of laboratory analysis (Figure 1), the highest total chlorophyll was noted in the control plants (0 Gy; 0.600 mol/m^2), whereas the least was produced by treatment with 75 Gy (0.209 mol/m^2). Irradiation rate of 30 Gy resulted in the highest anthocyanins (0.602 mol/m^2) and carotenoids (0.286 mol/m^2). The lowest anthocyanin was produced by treatment with irradiation of 60 Gy (0.137 mol/m^2), whereas the lowest carotenoids was found in treatment with 105 Gy (0.106 mol/m^2). The content of anthocyanins and carotenoids with irradiation treatments of 15, 30, and 45 Gy exceeded the content of anthocyanins and carotenoids in control plants (0 Gy).

3.2. Paradermal slice of leaf

The observations of leaves in the paradermal slice (Figure 2) and the results of analysis of variance (Table 1) show alterations in the anatomy of irradiated handeuleum leaf. Table 1 shows that the highest number of epidermal cells was found in plants irradiated with gamma rays at the rate of 60 Gy, which amounted to 303.6. The lowest number of epidermal cells was 223.8, found in plants irradiated with gamma rays at the rate of 75 Gy.

Plants irradiated with gamma rays at the rate of 30 Gy have the highest number of stomata of 111.0. The lowest number of stomata was found in plants treated with 90 Gy of gamma rays, which amounted to 63.0. The highest stomatal index (0.3) was produced by gamma ray irradiation treatment at 45 Gy. The smallest stomatal index (0.2) was found in plants irradiated with gamma rays at 90 Gy. The highest stomatal density (571.0) was generated by the treatment with gamma ray irradiation rate of 30 Gy. The smallest stomatal density (321.6) was produced by gamma ray irradiation rate of 90 Gy.

3.3. Transversal slice of leaf

The handeuleum leaf structure (transversal slices) is composed of layers of cuticle, upper epidermis, two layers of palisade, sponges, and lower epidermis (Figure 3). Figure 3 shows that chlorophyll and anthocyanin are generally found in the cells of palisade and a bit of sponge cells; the cell is marked with green and red. However, the plants irradiated with gamma ray at the rate of 60, 75, 90, and 105 Gy have less chlorophyll and anthocyanin content. As seen in Figure 4, the leaves are slight colored. The higher the rate of irradiation, the less the anthocyanin content is, as represented by the red color.

Based on the results showed in Table 2, all variables of transversal leaf anatomy (thickness of leaf, cuticle, upper epidermis, palisade, sponges, and lower epidermis) were significantly affected by gamma ray irradiation treatments. The thickest leaf was produced by gamma ray irradiation rate of 90 Gy (313.1 μm). The thinnest leaf was produced by a rate of 15 Gy (199.2 μm).

Gamma ray irradiation rate of 90 Gy resulted in the thickest cuticle (20.29 nm). The thinnest cuticle was produced by gamma ray irradiation rate of 0 Gy (5.9 nm). The thickest upper epidermis was produced by gamma ray irradiation rate of 105 Gy (43.7 nm), whereas the thinnest epidermis was produced by gamma ray irradiation rate of 0 Gy (14.8 nm).

The longest palisade was generated by the treatment of gamma ray irradiation rate of 75 Gy (92.3 nm), whereas the shortest palisade was produced by gamma ray irradiation rate of 15 Gy (37.5 nm). The thickest sponge was produced by gamma ray irradiation rate of 90 Gy (63.6 nm), whereas the thinnest sponge was produced by a treatment rate of 0 Gy (16.2 nm). For lower epidermis, the thickest was produced by gamma ray irradiation rate of 45 Gy (37.4 nm), whereas the thinnest was produced by a treatment rate of 0 Gy (15.3 nm).

4. Discussion

Our study generally resulted in high observation values when handeuleum leaves were treated with a low rate of gamma irradiation (15, 30, and 45 Gy) and degradation of plants when treated with a high rate of gamma irradiation (60, 75, 90, and 105 Gy), in comparison to controls.

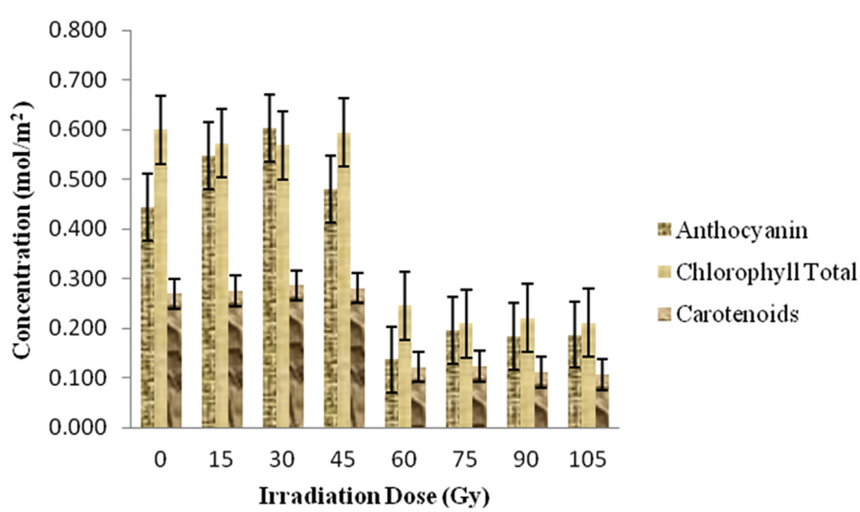

Figure 1. Comparison of anthocyanin pigment, total chlorophyll, and carotenoid contents in handeuleum plant (Bogor accession) on various rates of irradiation.

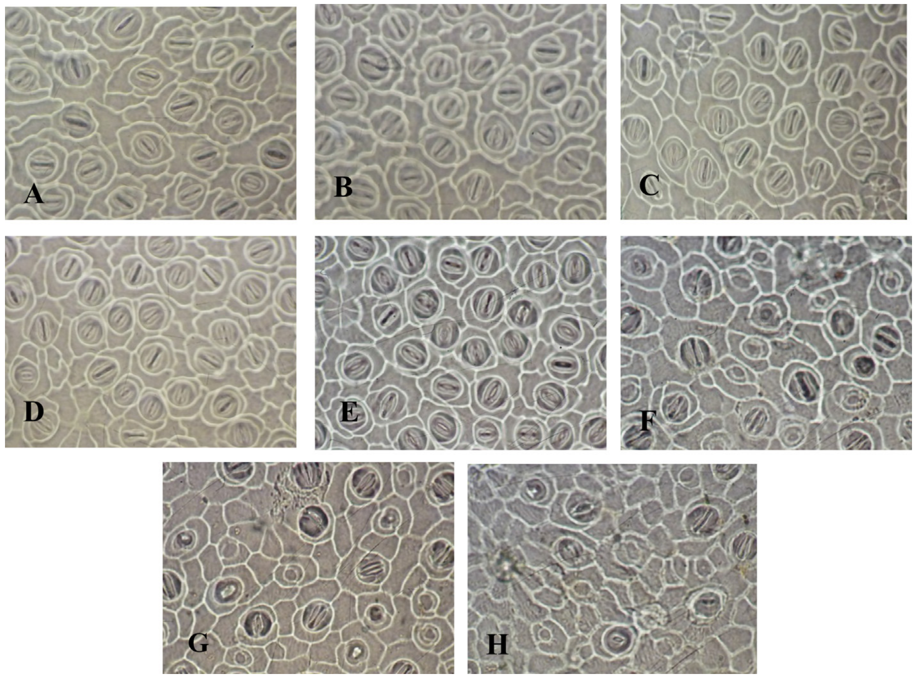

Figure 2. Comparison of leaf anatomical structure of handeuleum (Bogor accession) paradermal slices (400×) on various rates of treatment: 0 Gy (control; A), 15 Gy (B), 30 Gy (C), 45 Gy (D), 60 Gy (E), 75 Gy (F), 90 Gy (G), and 105 Gy (H). Higher rate of treatment shows less number of stomata.

Table 1. The average value of number of stomata, epidermal cell, stomatal index, and stomatal density of handeuleum leaf (Bogor accession) on various rates of gamma ray irradiation treatment

Irradiation dose (Gy)	Variables							
	Number of epidermis cell		Number of stomata		Stomatal index		Stomatal density (total/ mm^2)	
0	239.8 ± 27.8	bc	94.7 ± 7.7	bc	0.3 ± 0.03	a	482.7±39.0	bc
15	256.6 ± 17.4	b	108. ± 15.7	ab	0.3 ± 0.03	a	554.3 ± 79.8	a
30	284.9 ± 20.8	a	112.1 ± 8.8	a	0.3 ± 0.02	a	571.0 ± 44.9	a
45	248.1 ± 18.0	bc	111.4 ± 8.6	a	0.3 ± 0.02	a	567.6 ± 43.7	a
60	303.6 ± 35.2	a	104.5 ± 7.2	ab	0.2 ± 0.05	b	532.5 ± 87.9	ab
75	223.8 ± 2.1	c	89.0 ± 8.5	c	0.3 ± 0.02	a	453.5 ± 43.5	c
90	229.7 ± 42.6	bc	63.0 ± 19.3	d	0.2 ± 0.07	b	321.0 ± 98.4	d
105	243.2 ± 30.3	bc	95.2 ± 8.9	bc	0.3 ± 0.03	a	485.2 ± 45.5	bc

Note: Figures followed by the same letter in the same column of variables indicate no significant different in Duncan 5% test value. The values are presented with standard deviation.

Several studies on the stimulatory effects using gamma irradiation at low and high rates showed depression of phytochemical contents and anatomy of leaves (Jan et al. 2011; Widiastuti et al. 2010). However, Alikamanoglu et al. (2011) observed contradictory results that nonirradiated leaves showed an increase in phytochemical contents. Widiastuti et al. (2010) showed that the organizational structure of the leaf cells of mangosteen is very useful for understanding the morphogenetic changes in plants due to irradiation treatment.

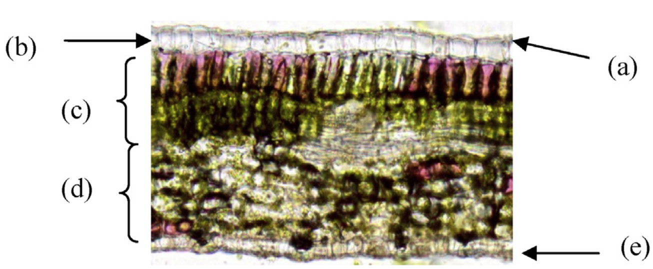

Figure 3. Transversal slices of handeuleum leaves (Bogor accession): cuticle (A), upper epidermis (B), palisade (C), sponges (D), and lower epidermis (E).

In our study, cuttings irradiated at high rate showed a delay in emergence of buds due to stress caused by gamma irradiation. There were no new leaves, and at the end of the study, the plants died. The difference of leaf and stem color between the control plant (0 Gy) and gamma-irradiated plants was strongly associated with anthocyanin, chlorophyll, and carotenoid content (Figure 1). Higher anthocyanin and carotenoid content was found in plants irradiated at low rate (15, 30, and 45 Gy) compared with controls, whereas the content of anthocyanins, total chlorophyll, and carotenoids decreased drastically in plants irradiated at high rate (60, 75, 90, and 105 Gy). The highest total chlorophyll content was produced by the control plants.

Jan et al. (2013) stated that low-rate gamma irradiation (10 kGy) in *Cullen corylifolium* (L.) can stimulate photosynthetic pigment system, where there is a significant increase in total chlorophyll to 71.66% when compared with controls, whereas high rates (15 and 20 kGy) reduce total chlorophyll. The carotenoid content continues to increase over increasing rates of gamma ray irradiation. Mohajer et al. (2014) explained that the gamma ray irradiation rate of 30, 60, 90, and 120 Gy increased the content of chlorophyll a, chlorophyll b, and carotenoids of *Onobrychis viciifolia* Scop. when compared with

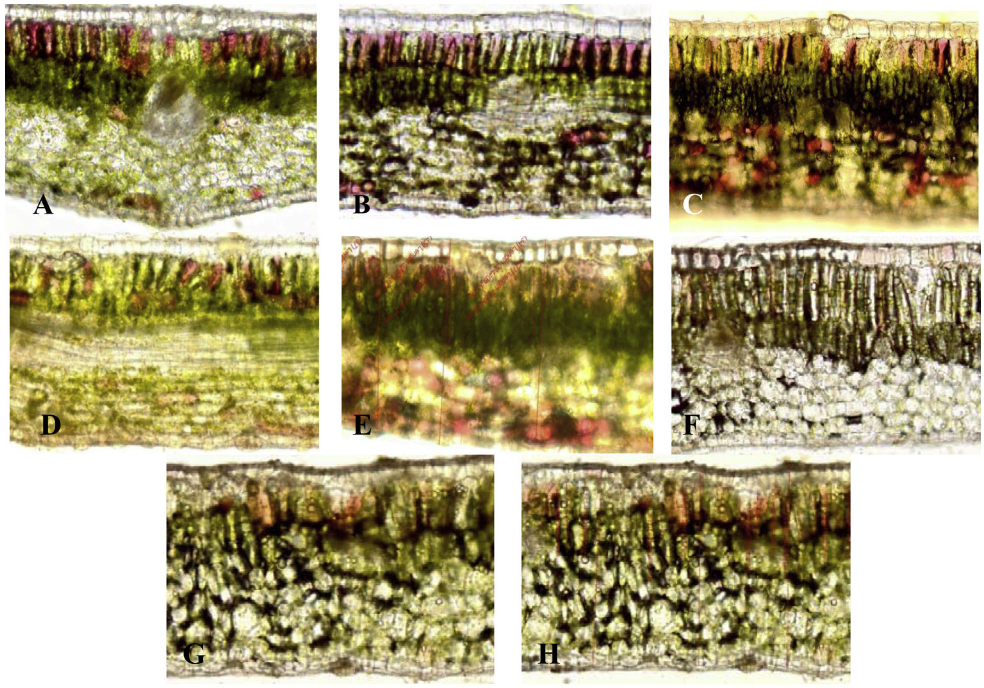

Figure 4. Comparison of leaf anatomical structure of handeuleum (transversal slices; Bogor accession): 0 Gy (control; A), 15 Gy (B), 30 Gy (C), 45 Gy (D), 60 Gy (E), 75 Gy (F), 90 Gy (G), and 105 Gy (H). Higher rate of irradiation shows smaller anthocyanin content which is represented by the red color.

Table 2. The average value of thickness of leaves, cuticle, upper epidermis, length of palisade, thickness of sponges, and lower epidermis of handeuleum leaves (Bogor accession) on various rates of gamma ray irradiation treatment

Irradiation dose (Gy)	Variables											
	Thickness of leaf (μm)		Thickness of cuticle (nm)		Thickness of upper epidermis (nm)		Length of palisade (nm)		Thickness of sponge (nm)		Thickness of lower epidermis (nm)	
0	225.2 ± 286.4	b	6.0 ± 1.0	e	14.8 ± 2.3	e	40.3 ± 584.7	d	16.2 ± 1.7	f	15.3 ± 1.6	d
15	199.2 ± 703.8	b	6.6 ± 1.5	de	30.5 ± 2.7	d	37.5 ± 647.1	d	31.8 ± 1.5	e	32.2 ± 4.3	b
30	230.8 ± 426.9	b	8.1 ± 1.1	d	27.7 ± 7.2	d	40.3 ± 644.8	d	34.4 ± 6.3	de	31.9 ± 4.7	b
45	236.3 ± 262.3	b	14.2 ± 1.7	c	38.7 ± 3.3	b	53.5 ± 146.0	c	38.2 ± 5.5	cd	37.4 ± 2.6	a
60	238.0 ± 216.6	b	13.0 ± 1.3	c	33.7 ± 5.0	c	81.0 ± 503.0	ab	35.8 ± 5.6	cde	24.6 ± 4.2	c
75	306.8 ± 993.2	a	18.9 ± 3.9	a	43.2 ± 3.3	a	92.3 ± 192.5	a	54.6 ± 7.5	b	26.7 ± 2.8	c
90	305.8 ± 124.8	a	20.3 ± 2.5	a	35.8 ± 3.6	bc	77.2 ± 107.8	b	63.6 ± 10.5	a	36.7 ± 3.7	a
105	313.1 ± 226.4	a	16.6 ± 4.3	b	43.7 ± 6.8	a	82.6 ± 539.6	ab	40.0 ± 8.8	c	26.0 ± 4.2	c

Note: Figures followed by the same letter in the same column of variables indicate no significant different in Duncan 5% test value. The value are presented with standard deviation.

controls, whereas the anthocyanin content decreased with the increasing rate of irradiation. Wi et al. (2007) suggested that chloroplasts are organelles that are very sensitive to irradiation with high doses of gamma rays, whereas the low-rate irradiation does not cause alterations in the structure of chloroplasts.

Handeuleum leaves have stomata located on the lower surface of the leaf. Based on observation of leaves, paradermal slice (Figure 2) and analysis of variance (Table 1) show that an alteration occurred in the irradiated handeuleum leaf anatomy. Generally, low-rate irradiation produces higher observation values than controls. A rate of 30 Gy resulted in the highest number of stomata and stomatal density, and the highest stomatal index was produced by a rate of 45 Gy. A rate of 60 Gy resulted in the highest number of epidermis compared with controls. The study by Widiastuti et al. (2010) on gamma ray irradiation of mangosteen showed changes in the size of the leaves, which are caused by changes in the number and/or size of the cells.

Handeuleum leaf structure belongs to the dorsiventral type because its palisade tissue is in between the upper epidermal tissue and spongy tissue (Figure 3). The high values of the variables of leaf anatomy (cuticle layer, upper epidermis, two layers of palisade, sponges) irradiated with high rate of gamma ray (60, 75, 90, and 105 Gy) caused stiff, hard, and frangible leaf texture. Irradiation using gamma rays caused changes both at the tissue and cellular level.

Sakr et al. (2013) stated that the gamma ray irradiation rate of 5, 10, and 15 Gy resized the epidermis, mesophyll, and the diameter of transport vessel of leaves, stems, and root organs. The anatomical changes are generally followed by changes in physiological activity. Gamma ray irradiation caused a color change in leaves and stems of handeuleum. Plants at high rates of irradiation (60, 75, and 90 Gy) had a purplish green leaves, whereas leaves turn into green at the rate of 105 Gy. Similarly, the stem color of plants irradiated at the rate of 75, 90, and 105 Gy was green. The purplish red color produced in the handeuleum plant was caused by the anthocyanin pigment.

Chlorophyll produces green color; anthocyanins produce a wide range of colors, ranging from orange/red to purple/blue, whereas carotene produces yellow/red (Tanaka et al. 2008). Anthocyanins have a wide distribution in the leaves and are present in the vacuole cells (Tanaka et al. 2008; Pfundel et al. 2006) and accumulate mainly in epidermis cells (Pfundel et al. 2006). Chlorophyll is synthesized in the chloroplast (Wi et al. 2007), carotenoids are synthesized in the chloroplast, and anthocyanins are synthesized in the cytosol (Tanaka et al. 2008). The photosynthetic pigments serve to protect plants from damage caused by irradiation. Based on the transverse cross sections of leaves (Figure 3), purple color seen on the leaves and stems is allegedly due to a more dispersed location of anthocyanin, which is located on the first layer of palisade. This determines the color of the leaves of handeuleum, particularly the visible ones. Our results showed that anthocyanin and chlorophyll content was nearly similar. The anthocyanins scattered in handeuleum leaves cover chlorophyll and carotenoids, which are located in chloroplasts in the second layer of palisade and sponge, resulting in greater role of anthocyanin in causing the handeuleum color (purple). The green color on the irradiated handeuleum is allegedly because anthocyanin pigment is the first damaged pigment; this is because it lies on top of the cells that contain chlorophyll and carotenoids. Thus, by the destruction of the anthocyanin, the color of the handeuleum leaf is then determined by chlorophyll and carotenoid pigments. Increasing height of palisade and thickness of sponge in plants irradiated with high rates of gamma are not followed by increasing chlorophyll and anthocyanin contained therein. The chlorophyll and anthocyanin content is allegedly degraded in irradiated plants at the rates of 60, 75, 90, and 105 Gy.

Research conducted by Kim et al. (2009) suggests that the gamma ray irradiation at the rate of 50 Gy increases chlorophyll and carotenoid content in *Arabidopsis* leaves when compared with the control because the process of transcription of genes involved in chlorophyll and anthocyanin anabolism increases when compared with the control. According to Alikamanoglu et al. (2011), after getting a high rate of gamma ray irradiation (100–500 Gy), chlorophyll content degraded in soybean plants. Gamma ray irradiation might affect the primary metabolism, which then influences the level of secondary metabolism (Jan et al. 2013). Wi et al. (2007) stated that gamma rays induce production of reactive oxygen species (ROS), which can damage or modify an important component of the plant cell, causing changes in morphology, anatomy, biochemistry, and physiology depending on the level of irradiation. These effects include changes in cellular structure and metabolism of plants, photosynthesis, antioxidative system, and accumulation of phenolic compounds.

Gamma ray irradiation works by ionizing the atoms in the tissue by removing electrons from the atom (Aisyah et al. 2009). It is called ionization, as passing through the material, it will release a separate energy (Ismachin 2007). Ionization process formed ionized water molecules ($H_2O^{\circ-}$) and free electrons (e^-). Water molecules are ionized and then generate $^{\circ}OH$ radicals and H°, which, if binded with oxygen, produce hydrogen peroxide (H_2O_2). While the free electrons (e^-) polarize water molecules (which are found in plenty in plant tissues) into solvated electron/hydrated electrons (e^{-aq}), which, when hydrated electron binds with oxygen, produce superoxide anion ($O_2^{\circ-}$) (Esnault et al. 2010). Primary free radicals ($^{\circ}OH$, H°) and secondary free radicals (H_2O_2, $O_2^{\circ-}$) are usually called as ROS. According to Gill and Tuteja (2010), ROS H_2O_2 has a long half-life in the tissue. In addition, these ROS may be able to inactivate the enzyme by oxidizing the thiol groups; thus H_2O_2 causes major damage in irradiated plants. ROS cause damage to lipids, proteins, carbohydrates, and DNA that will lead to cell death. DNA alteration leads to alterations in the genes and therefore automatically changes reactions controlled by these genes; it eventually will lead to alterations in morphology, anatomy, biochemistry, and physiology of plants (Wi et al. 2007).

Results obtained from this research showed that there is a connection between increasing rate of gamma irradiation with alteration structure of leaf anatomy and phytochemical content of handeuleum. Low-rate gamma irradiation (30 Gy) produced high content of anthocyanin and carotenoids and also the produced highest number of stomata and stomatal density compared with control plants. Low-rate gamma irradiation (45 Gy) produced the highest stomatal index. High-rate gamma irradiation (75, 90, and 105 Gy) produced rigid, thick, and frangible leaves; this was because palisade, sponges, and upper epidermis were bigger than those in control plants.

Conflict of interest

There is no conflict interest regarding this paper.

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