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Physiological Responses of *Citrus sinensis* to Gamma Irradiation

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Abstract: Gamma irradiation is one of the physical mutagens that widely used for mutation breeding, food sterilisation and medicinal healing. In the present study, in vitro mutagenesis techniques were applied to investigate the effects of gamma irradiation at 0, 10, 20, 30, 40 and 50Gy on physiological changes in *Citrus sinensis*. Studies revealed that based on the height increment, the LD_{50} (gamma doses that killed 50% of the plantlets) of the plantlets were achieved at 27Gy. Biochemical differentiation based on total soluble protein content revealed that plantlet irradiated at 50Gy contain the highest amount of total soluble protein, 21.03±1.82mg/gFW, whereas only 14.49±4.04mg/gFW of total soluble protein was detected in 10Gy. However, the highest amount of specific activity of peroxidase was obtained in plantlets irradiated at 50Gy. Conversely, non-irradiated plantlets demonstrated the highest amount of chlorophyll content as compared to plantlet irradiated at 10, 20, 30, 40 and 50Gy. In addition, the amount of chlorophyll a was to be higher than chlorophyll b in both irradiated and non-irradiated plantlets.

Key words: *Citrus sinensis · In vitro mutagenesis · Gamma irradiation*

INTRODUCTION

Gamma irradiation has been widely applied in medicine and biology in terms of biological effects induced by a counter intuitive switch-over from low doses stimulation to high-doses inhibition [1]. Previous studies have shown that relatively low-doses ionizing irradiation on plants and photosynthetic microorganisms are manifested as accelerated cell proliferation, germination rate, cell growth, enzyme activity, stress resistance and crop yields [2]. In vitro mutagenesis is a combination of in vitro culture and mutation induction which provides the opportunity to increase variability of an economically important cultivar or used on plants in developing varieties that are agriculturally and have high productivity potential [3]. Traits induced by mutagenesis include plant size, blooming time and fruit ripening, fruit colours, self-compatibility, self-thinning and resistance to pathogens [4]. Induced mutation technique is a valuable tool but not yet fully exploited in fruit breeding [5]. Tissue culture makes it more efficient by allowing the handling of large populations and by increasing mutation induction efficiency, possibility of mutant recovery and speediness of cloning selected variants [6].

Citrus sinensis, is a member of Rutaceae family (citrus family) and it is commonly known as 'sweet orange' or 'navel orange' [7]. *C. sinensis* fresh fruit is one of the major export crops in global trade whereby it generates about 105 billions USD/ year [8]. Unfortunately, citrus is attacked by several plant pathogens that affect its fruit quality. Major post-harvest losses have been recorded on the export markets associated with a range of pathogens. Throughout the world market, one of the most crucial factors affecting the marketing of citrus fruits are loss due to citrus black spot, Tristeza disease and citrus greening disease [9]. According to Khan and Roose [10], citrus is a plant that have a long juvenility period and the breeding of citrus cultivars by conventional methods are restricted by the complication of their genetic systems [11]. To date there is no major report stating the use of gamma irradiation as a physical mutagen to alter the physiological characteristics of *C. sinensis*. Thus, the aim of the present investigation was conducted to tackle this issue by performing the physiological studies on *C. sinensis*, after exposure to different doses of gamma rays.

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MATERIALS AND METHODS

Plant Materials: The seeds of *C. sinensis* were cultured in Murashige and Skoog (MS) medium [12].

Gamma Irradiation: The seeds were cultured for two days prior to gamma irradiation. Gamma irradiation treatment was carried out using Caesium-137 source at a doses rate of 4.49397712KGy/hr in the Malaysia Nuclear Agency at Bangi, Selangor, Malaysia. The doses applied in this study were 0Gy, 10Gy, 20Gy, 30Gy, 40Gy and 50Gy. After irradiation, the seeds were transferred and maintained in a fresh new MS basal medium and were maintained at 25±2°C with the photoperiod of 16 hours light and 8 hours dark in a culture room.

Radiation Sensitivity Test: Radiation sensitivity test was conducted on *C. sinensis* based on the height increment after three weeks of exposure to the gamma irradiation. The LD_{50} (LD = "lethal doses") was used to determine the gamma doses that killed half (50%) of the plantlets.

Sample Extraction: The irradiated and non-irradiated plantlets were homogenized in ice bath with protein extraction buffer of the ratio of 1g sample to 3mL of protein extraction buffer. Crude extracts were transferred to 1.5mL Eppendorf tubes followed by centrifugation at 12,000rpm for 20 minutes at 4°C. The resulting supernatant was collected and was used to determine the total soluble protein and specific activity of peroxidase of irradiated and non-irradiated plantlets of *C. sinensis*.

Determination of Total Soluble Protein: Total soluble protein content of the irradiated and non-irradiated plantlets was determined using the Bradford method [13]. In order to determine the total soluble protein content, 20µL of the sample extract was added into 80µL of protein extraction buffer and 5mL of protein reagent. The mixture was mixed by vortexing. In contrast to sample solution, 20µL of double distilled water and 80µL of protein extraction buffer with 5mL of protein reagent was used as the blank. Absorbance at 595nm was determined using the spectrophotometer (Bio-Rad Smartspec plus, USA). The absorbance was compared with the standard curve plotted using bovine serum albumin (BSA) (Sigma Aldrich, USA) as the standard at the concentrations of 0, 100, 300, 500, 700 and 1000µg/mL and further expressed in milligram per gram fresh weight of plant material.

Determination of Specific Activity of Peroxidase: The specific activity of peroxidase of the irradiated and non-irradiated plantlets were measured and determined by Kokkinakis and Brooks method [14]. Activity of peroxidase was determined based on the appearance of brown colours resulting from guaiacol oxidation in the presence of hydrogen peroxide. Reaction mixture consisted of 50µL sample extract, 2.6mL of 0.1M sodium phosphate buffer at pH 6.1 and 0.3mL of 1% guaiacol (Fisher, USA) was added into the solution. A total of 0.3mL of 30% H_2O_2 (Fisher, USA) was added prior to reaction. Changes in absorbance at 420nm were followed for three minutes using a spectrophotometer (Bio-Rad smartspec plus, USA). Peroxidase activity was calculated using the formula below and expressed in unit/mg protein:

$$\text{Specific activity of peroxidase} = \frac{\text{Total activies of the sample}}{\text{protein contant of the sample}}$$

$$\text{Total activities} = \frac{\Delta \text{ Abs x dilution factor} \times 1000}{\text{volume of enzyme used in the assay}}$$

Determination of Chlorophyll Content: Chlorophyll content of irradiated and non-irradiated plantlets was determined using the Lichtenthaler [15] method. Irradiated and non-irradiated plantlets were added to a pre-chilled mortar in an ice bath. The plantlets were extracted with 10mL of 80% (v/v) acetone (Merck, USA) at the ratio of 1g sample to 2g of calcium carbonate ($CaCO_3$) (Spectrum, USA). The sample extract was collected and filtered with Buchner Funnel through Double Ring filter paper. The extraction volume was topped up to 50mL with 80% (v/v) acetone. The sample extract was determined at 646nm and 663nm in a Genesys 20 spectrophotometer (Bio-Rad smartspec plus, USA). The chlorophyll a (C_a) and chlorophyll b (C_b) content in milligram per liter was determined according to the formulae below and further expressed in milligram per gram fresh weight of plant material:

Chlorophyll a, $C_a = 12.25\ (A_{663}) - 2.79\ (A_{646})$
Chlorophyll b, $C_b = 21.50\ (A_{646}) - 5.10\ (A_{663})$
Total chlorophyll, $C_{a+b} = 7.15\ (A_{663}) + 18.71\ (A_{646})$

Statistical Analysis: In this study, three replicates were conducted for physiological changes and the experiments were repeated twice. The results of physiological changes of irradiated and non-irradiated

plantlets of *C. sinensis* were subjected to statistical analysis one-way ANOVA and Tukey's Honestly Significant Different (HSD) test using SPSS software (version 15.0) (SPSS Inc. USA) at $p<0.05$.

RESULTS AND DISCUSSION

Radiation Sensitivity Test: The radiation sensitivity of *Citrus sinensis* was compared by measuring the height increment of irradiated and non-irradiated plantlets after three weeks of irradiation. The plantlet height was inhibited with increasing doses of gamma irradiation as illustrated in Fig. 1. For the height percentage of irradiated plantlets to reach 50%, the gamma doses administered was 27Gy as interpolated from the graph in Fig. 1.

In this study, a noticeable reduction in radiation sensitivity was observed with increasing gamma doses. In a previous study, when seeds of red pepper (cv. Yeomyung and Joheung) were first gamma-irradiated, their resultant plant growth was stimulated at 2 to 8Gy but was hardly affected at 16Gy [16]. In contrast, the present data indicated the stimulation of plant growth at 10Gy but inhibition occurred above 10Gy. There were no much variations in growth between these two studies. This implies that irradiation increases plant sensitivity to gamma rays. This may be caused by the reduced amount of endogenous growth regulators, especially the cytokines, as a result of break down, or lack of synthesis, due to irradiation [17].

Determination of Total Soluble Protein: Total soluble protein content of the *C. sinensis* showed some differences depending on the gamma irradiated doses. Biochemical differentiation based on total soluble protein content revealed that plantlet irradiated at 50Gy contain the highest amount of total soluble protein, 21.03±1.82mg/gFW, whereas only 14.49±4.04mg/gFW of total soluble protein was detected in 10Gy (Fig. 2). Comparing total protein content of control plantlets and 10Gy irradiated plantlets, the control plantlets exhibited significantly greater than those of 10Gy irradiated plantlets. Plantlets irradiated at 10Gy exhibited a total soluble protein content of 14.49±4.04mg/gFW which was 20.90% lower than that of the non-irradiated plantlets, 18.32±1.39mg/gFW. However, there was no significant difference among the plantlets irradiated with 20, 30, 40 and 50Gy. Studies also revealed that plantlets irradiated at 20, 30, 40 and 50Gy recorded total soluble protein content of 17.50±1.68 mg/gFW, 18.86±3.32 mg/gFW, 20.57±2.52 mg/gFW, 21.03±1.82 mg/gFW, respectively that were not significantly differed from one to another.

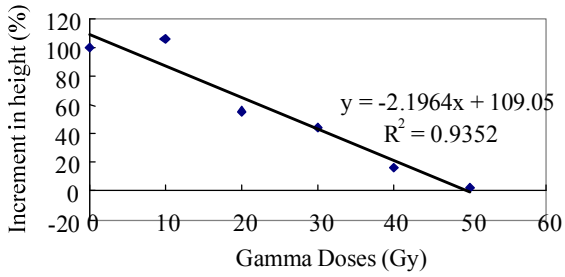

Fig. 1: Radiation sensitivity test for *C. sinensis*

In this study, it was found that there was an irregular distribution of total soluble protein content in irradiated and non-irradiated plantlets. According to the results obtained in the present study, it was observed that plantlets irradiated at high doses (30, 40 and 50Gy) displayed a higher total soluble protein content as compared to their non-irradiated plantlets. Conversely, it was observed that plantlets irradiated at relatively low doses (10 and 20Gy) caused a reduction of total soluble protein content.

Humera [18] stated that the stress reaction of plants often results in the alteration of protein metabolism. Several proteins are synthesized and accumulated in plant tissues under a range of stress conditions. Such proteins, referred to as stress proteins, have been noted to be induced in response to several stress factors. The most crucial function of plant cell is to respond to gamma stress by developing defenses mechanisms. This defense was brought about by alteration in the pattern of gene expression [19]. This led to modulation of certain metabolic and defensive pathways [20]. Owing to gene expression altered under gamma stress, qualitative and quantitative changes in total soluble protein content was obvious [19]. These proteins might play a role in signal transduction, anti-oxidative defense, anti-freezing, heat shock, metal binding, anti-pathogenesis or osmolyte synthesis which were essential to a plant's function and growth [21].

In general, radiation causes the irreversible changes of protein conformation at the molecular level by breakage of covalent bonds of polypeptide chains [22]. Fragmentation involves reaction of alpha-carbon radicals with oxygen to form peroxyl radicals which decompose to fragment the polypeptide chain at the alpha carbon [23]. Hydroxyl radical and superoxide anion radical generated by radiation could modify primary structure of

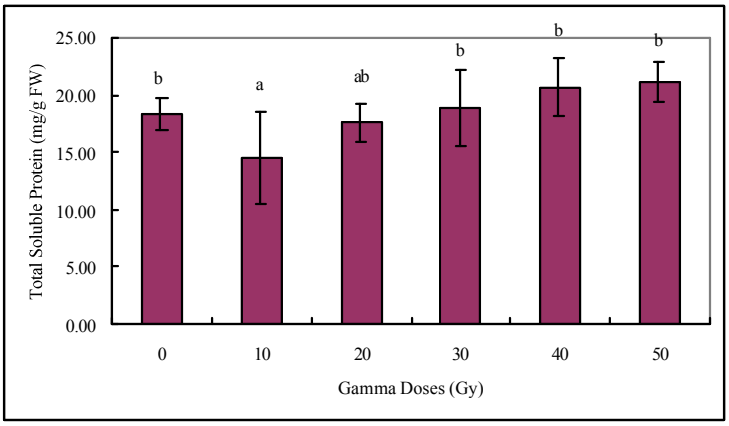

Fig. 2: Effects of gamma irradiation on total soluble protein content of *C. sinensis* after three weeks of culture. Mean with different letter (s) are significantly differed between treatments by the Tukey's HSD test ($p<0.05$). Error bars indicate the mean ± standard deviation.

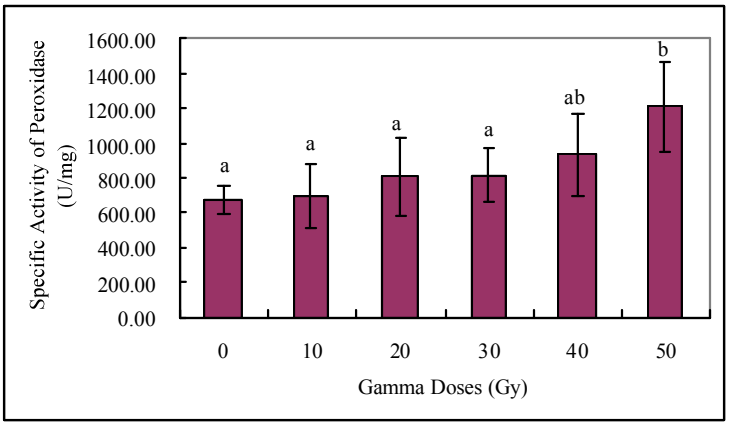

Fig. 3: Effects of gamma irradiation on specific activity of peroxidase of *C. sinensis* after three weeks of culture. Mean with different letter (s) are significantly differed between treatments by the Tukey's HSD test ($p<0.05$). Error bars indicate the mean ± standard deviation.

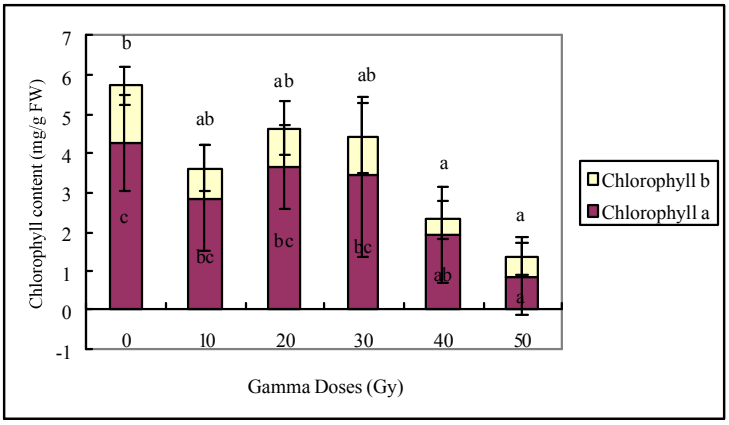

Fig. 4: Effects of gamma irradiation on chlorophyll content of *C. sinensis* after three weeks of culture. Mean with different letter (s) are significantly differed between treatments by the Tukey's H SD test ($p<0.05$). Error bars indicate the mean ± standard deviation

proteins, which resulted in changes of molecular weight distribution [24]. Besides fragmentation, aggregation of protein fragmented is also observed. There have been reports on aggregation and cross-linking of proteins by irradiation [25]. Covalent cross-linkages are formed between soluble proteins and between peptides and proteins [24].

A survey of the literature shows that there was no consistent report regarding the effect of irradiation on the nucleic acids, protein and nitrogen. Some investigators have observed slight depression or increase, while others reported no significant changes. Constantin and Love [26] observed a slight decrease in protein in gamma-irradiated *Vigna sinensis* seedlings. They quoted Pollard [27], who postulated that irradiation stops the DNA transcription and lead to a decrease in protein synthesis and growth. Pollard [27] reported that in *Chlorella pyrenoidosa,* the cells exposed for 3 days to gamma-irradiation (7200r/day) increased in volume and had increased amounts of DNA and protein (per cell). Indications are that irradiation can effect protein directly and indirectly [28] and that protein synthesis is inhibited [29], unaffected or enhanced [30].

Determination of Specific Activity of Peroxidase: Specify activity of peroxidase of irradiated and non-irradiated plantlets were obtained after 3 weeks of culture. According to Fig. 3, the highest specific activity of peroxidase (1208.92±257.84 U/mg) was recorded in 50Gy irradiated plantlets followed by 934.72±239.71 U/mg in the 40Gy irradiated plantlets. Specific activity of peroxidase for plantlets irradiated with 50Gy was significantly greater than the non-irradiated plantlets. Plantlets irradiated at 10, 20, 30Gy exhibited specific activity of peroxidase of 690.55±182.24 U/mg, 806.90±217.45 U/mg, 812.69±158.35 U/mg which were not significantly differed than the non-irradiated plantlets, 676.24±80.00 U/mg. Plantlets irradiated at 10, 20, 30, 40 and 50Gy recorded specific activity of peroxidase which was an excess of 2.0, 16.2, 16.8 and 44.1%, respectively over the non-irradiated plantlets. Irradiation doses at 10, 20, 30 and 40Gy produced significantly higher specific activity of peroxidase than those of the non-irradiated plantlets but there was no significant difference between each other.

Peroxidase (POD) among antioxidant enzymes plays an important role of hydrogen peroxide (H_2O_2) detoxification in cells, thereby protecting cellular components such as proteins and lipids against oxidation [31]. The PODs are also required essentially for a variety of cellular functions such as lignification, suberization, cell elongation, growth, regulation of cell wall biosynthesis and plasticity [32-34]. Thus, it is very important to analyze changes in the spatial distribution and the amount of POD after gamma irradiation. In the study by Wi *et al.* [35], the induction of POD by the irradiation would be one of the defense systems activated through the ROS-mediated cellular signaling. As observed in the present study, it was suggested that the increase in gamma doses corresponded to an increase in specific activity of peroxidase. Likewise, the result in this study was supported by Sah *et al*. [36] whereby enhanced peroxidase activity in barley was observed after irradiation of seeds with gamma-irradiation. Wang *et al* .[37] used peroxidase isozymes for resistance to powdery mildew in wheat. Similarly Qin *et al*. [38] noticed a change in the peroxidase activity in *Lathyrus sativus* plants after treatment of seeds with gamma rays and EMS. Enhancement in peroxidase activity by radiation has also been reported by Omar [17] in sunflower.

In fact, it has been suggested by Rumaih [31], in the study of the effects of ionizing radiation on trigonella species that the activity and isozyme patterns of peroxidase in *Nicotiana debneyi* and *Nicotiana tabacum* were increased in response to gamma irradiation treatment. Apart from that, Singh *et al*. [39] also noticed the induction of peroxidase activity in two sugar cane varieties grown under gamma rays. Meanwhile, the activities of peroxidase in radish (*Raphanus sativus*) leaves were enhanced by gamma irradiation at 10Gy [40]. It also has been indicated by Stoeva [41] that gamma irradiation enhanced peroxidase activity of two *Phaseolus vulgaris* cultivars [42].

Determination of Chlorophyll Content: In this study, biochemical differentiations based on chlorophyll content of irradiated and non-irradiated plantlets were obtained after 3 weeks of culture. As illustrated in Fig. 4, an increase in chlorophyll a, b and total chlorophyll levels was observed in non-irradiated plantlets. All the irradiated plantlets exhibited lower amount of chlorophyll a and b as compared to the non-irradiated plantlets, 5.70±1.68mg/gFW. Plantlets irradiated at 10, 20 and 30Gy exhibited total chlorophyll content of 3.61±1.81 mg/gFW, 4.64±1.71 mg/gFW, 4.40±2.79 mg/gFW respectively but were not significantly differed from one another. Plantlets irradiated at 40 and 50Gy exhibited total chlorophyll content of 2.33±1.59 mg/gFW, 1.32±1.03 mg/gFW, respectively which was significantly differed as compared to the non-irradiated plantlets, 5.70±1.68 mg/gFW. The lowest total chlorophyll content was observed in plantlet irradiated at 50Gy. Furthermore, plantlets irradiated at

high doses, 40 and 50Gy demonstrated a drastic reduction 59.1 and 76.9%, respectively in total chlorophyll content as compared to the non-irradiated plantlets. Obviously, the content of chlorophyll a was higher than chlorophyll b in both irradiated and non-irradiated plantlets (Fig. 4).

In present study, the chlorophyll content showed irregular distribution among the irradiated plantlets. Lower chlorophyll content was obtained from irradiated plantlets as compared to non-irradiated plantlets. This result was supported by Kim *et al.* [16] whereby chlorophyll is virtually insensitive to low doses gamma irradiation. In addition to that, it can be observed that the concentration of chlorophyll a was relatively higher than chlorophyll b in both irradiated and non-irradiated plantlets. In previous study, it has been reported that gamma irradiation resulted in greater reduction in the amount of chlorophyll b as opposed to chlorophyll a [42]. Furthermore, the reduction in chlorophyll b is due to more selective destruction of chlorophyll b biosynthesis or degradation of chlorophyll b precursors [43]. Abu *et al.* [44] stated that an increase in chlorophyll a, b and total chlorophyll levels was observed in *Paulownia tomentosa* plants that were exposed to gamma irradiation.

In future prospects, the effects of gamma irradiation on *C. sinensis* could be carried out to enable the production of a mutant with superior qualities to produce vast amounts of medicinally important secondary metabolites such as flavonoids. Besides that, agarose gel electrophoresis on DNA and RNA could be performed on gamma irradiated *C. sinensis* to study the difference in gene expression pattern of the irradiated plant species.

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