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Morphological and Physiological Responses of *Orthosiphon stamineus* Callus to Gamma Irradiation at Different Doses

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Abstract: In this mutagenic study, different doses of gamma rays (10, 20, 30, 40 and 50 Grays) have been used to induce mutations by physical means to *Orthosiphon stamineus* callus. No obvious changes were observed in terms of colour and morphology of the callus. After three weeks of irradiation, various biochemical studies such as the chlorophyll content, total soluble protein and rosmarinic acid content as well as the specific peroxidase activity of the callus were conducted. Results showed a steady decrement of total soluble protein content with the increment of irradiation strength, with the non-irradiated sample demonstrated the highest total soluble protein content (6.31±1.64 mg/g FW). Similar trend was observed for specific peroxidase activity where the non-irradiated samples recorded the highest value of 1652.68±1160.78 units/mg soluble protein. Total chlorophyll content of gamma irradiated callus was also found to be lower compared to the non-irradiated callus. Nevertheless, there was an exception for callus irradiated at 30Gy which was found to be 15.62% higher in the total chlorophyll content compared to the non-irradiated callus. With higher irradiation doses, the production of rosmarinic acid was found to be higher where samples irradiated at 50Gy demonstrated the highest rosmarinic acid concentration (132.64±27.03 mg/g FW).

Key words: *Orthosiphon stamineus* · callus culture · Rosmarinic acid · *in vitro* mutagenesis · Gamma irradiation

INTRODUCTION

In vitro mutagenesis or also known as directed mutagenesis is defined as the process of generation of nucleotide changes in cloned genes by any one of several procedures, including site-specific and random mutagenesis [1]. Mutagenesis is a vital process in the improvement of food crops to enhance yields and facilitate cultivation which has been central to development in all societies [2]. Mutagenesis can be achieved via chemical, physical or biological means. Gamma irradiation is a kind of physical means which has been widely applied in medicine and biology in terms of biological effects induced by a counter-intuitive switch-over from low-dose stimulation to high-dose inhibition. Previous studies have shown that relatively low-dose ionizing radiation on plants and photosynthetic microorganisms are manifested as accelerated cell proliferation, germination rate, cell growth, enzyme activity, stress resistance and crop yields [3-8]. In a recent study, there have been a number of reports on the use of UV, low-energy ultrasound, hormone and feeding of precursors to regulate the production of secondary metabolites such as shikonin, anthraquinone, saponins, silymarin, anthocyanin in suspension culture system [9-12]. However there is very limited information on the enhancement of production of secondary metabolites induced by gamma radiation, especially rosmarinic acid, even though a variety of biological effects of gamma radiation have received considerable attention [3-4].

In recent times, plants are still an important source for the discovery of novel pharmacologically active compounds, with many blockbuster drugs being derived directly or indirectly from plants. Controlled growth systems (e.g. callus culture) will make it feasible to contemplate manipulation of phenotypic variation

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(via gamma irradiation) in the concentration of medicinally important compounds present at harvest where the aim is to increase potency, reduce toxin levels and increase uniformity and predictability of extracts. It is believed that *O. stamineus* available in Malaysia are unable to meet the market demand for the preparation of the herbal product [13]. Hence, there is an urgent need to apply non conventional methods for future commercial supply of this plant to meet the market demand. In view of *O. stamineus* is widely used for the treatments of various kidney and liver ailments, aiding with promising results done by previous studies on phytochemicals activities, this study was conducted to determine the effects of gamma irradiation at different doses on the morphological and several important physiological changes such as total soluble protein, specific activity of peroxidase, chlorophyll and rosmarinic acid content in the callus cultures of *O. stamineus*.

MATERIALS AND METHODS

Plant Materials: Six-week old *in vitro* plantlet of *Orthosiphon stamineus* was used as the plant material for the induction of callus. The *in vitro* plantlet was maintained in full strength MS medium supplemented with 3% (w/v) sucrose.

Medium Preparation: Full strength of Murashige and Skoog medium [14] was used in this study. The medium was prepared from each stock solution (×100) consisting of all the macronutrients, micronutrients, vitamins, FeNaEDTA and plant growth regulators. For callus induction purposes, 2,4-D (Sigma, USA) at the concentration of 3 mg/L was added into the medium. Sucrose at 3% (w/v) (Sigma, USA) was used as carbon source. Medium pH was then adjusted with pH meter to pH 5.7±0.1 with 0.1M of HCl (Sigma,USA) or NaOH (Sigma, USA). The medium was autoclaved at 121°C, 15 psi for 15 minutes. Then, the autoclaved medium was left to cool to 60°C to 70°C before the medium was distributed with a volume of 25 mL to each petri dish and sealed properly prior to the experiment.

Induction and Maintenance of Callus Culture: The six-week old healthy *in vitro* plantlet was chosen for the induction of callus culture. Approximately ten *in vitro* leaf explants were cut to the size of 5mm × 5mm and cultured in the petri dish containing MS medium supplemented with 3.0 mg/L 2,4-D. The cultures were then incubated at the photoperiod of 16 hours light and 8 hours dark under fluorescent lighting at the temperature of 25±1°C. Meanwhile, the maintenance of the callus was initiated by separating the callus from the explant. Only the healthy callus (friable and yellowish in colour) were transferred into the new fresh full strength MS medium containing 3.0 mg/L 2,4-D and regular subculturing process was conducted at two weeks interval. The third passage callus was used to initiate the gamma irradiation studies.

Gamma Irradiation: Gamma irradiation was done at Malaysian Nuclear Agency, located at Bangi, Selangor using Ceasium-137 as radiation source at a dosage rate of 4.640 kGy/hour. For the irradiation purposes, a total of 0.5g or 1.0g of two weeks old callus was exposed to gamma irradiation at the doses of 0, 10, 20, 30, 40 and 50Gy. The callus was transferred to fresh medium with the same composition directly after irradiation and further incubated at the photoperiod of 16 hours light and 8 hours dark under fluorescent lighting at the temperature of 25±1°C. Morphological and physiological responses of the callus towards gamma irradiation were observed after three weeks of irradiation.

Morphological Responses: Approximately 0.5g of callus was exposed to 0, 10, 20, 30, 40 and 50 Gy doses of irradiation. The morphological responses of the irradiated and non-irradiated calli of *O. stamineus* were observed continuously for three weeks. The observation made was based on three parameters; changes in fresh weight, morphology and colour.

Enzyme Extraction: Irradiated and non-irradiated callus were ground with protein extraction buffer at a ratio of 1g of callus to 3mL of extraction buffer using pestle and mortar pre-chilled in an ice bath. The enzyme extracts were then transferred into 15 mL centrifuge tube and centrifuged at 12,000 rpm, 4°C for 20 minutes. The supernatant was used to determine the total soluble protein content and specific activity of peroxidase of irradiated and non-irradiated callus of *O. stamineus*.

Determination of Total Soluble Protein Content: Total soluble protein content in irradiated and non-irradiated callus of *O. stamineus* was determined according to Bradford method [15] using bovine serum albumin (BSA) (Sigma, USA) as a standard. A total of 20µL of extracts and 80µL of protein extraction buffer together with 5µL of protein reagent were mixed and absorbance was measured using spectrophotometer (Bio-Rad, USA) at 595nm. A standard calibration curve was constructed by using

a known series of BSA concentrations at 0, 50, 100, 150, 200, 250, 300, 350 and 450μg/mL. The total soluble proteins present in the samples were estimated by comparing the absorbance with the standard curve and were further expressed in milligram per gram fresh weight (mg/g FW).

Determination of Specific Activity of Peroxidase: The same samples extracts used to determine total soluble protein content were used to determine the specific activity of peroxidase. The blank consisted of 2.6mL 0.1M sodium phosphate buffer (pH 6.1), 0.3mL 1% (v/v) guaiacol (Fisher, USA), 0.3mL 30% (v/v) hydrogen peroxide (Fisher, USA), together with 50μL of protein extraction buffer. A total of 50μL of sample extract together with 2.6mL 0.1M sodium phosphate buffer (pH 6.1), 0.3mL 1% (v/v) guaiacol (Fisher, USA) and 0.3mL 30% (v/v) hydrogen peroxide (Fisher, USA) were subjected to vortex. Absorbance was measured for 3 minutes using a spectrophotometer (Bio-Rad, USA) at 420nm. The initial absorbance and the maximum absorbance value were recorded. One unit of specific activity of peroxidase (U) is equivalent to the amount of enzyme used to reduce hydrogen peroxide in one minute per mg of soluble protein. The specific activity of peroxidise was calculated using the formula below [16]:

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

$$Where;\ \Delta Abs = Maximum\ absorbance\ value - Initial\ absorbance\ value$$

$$Specific\ activity\ of\ peroxidase\ (units/mg) = \frac{total\ activities}{protein\ content\ of\ the\ sample\ (mg)}$$

Determination of Chlorophyll Content: The chlorophyll was extracted according to Harborne Method [17]. Irradiated and non-irradiated callus were ground with calcium carbonate, $CaCO_3$ (Spectrum, USA) at the ratio of 1g: 3mL and 10mL of 80% (v/v) acetone was added for the extraction. The extract was then filtered with filter paper and washed with 5mL of 80% (v/v) acetone. The extraction volume was then made up to 50 mL using 80% (v/v) acetone. Absorbance of chlorophyll extract was measured using spectrophotometer (Bio-Rad, USA) at 645nm and 663nm. The chlorophyll content was determined using the Lichtenthaler method [18] expressed in milligram per liter and further expressed in milligram per gram fresh weight of plant material according to the formulae below:

$$Chlorophyll\ a, C_a\ (mg/L) = 12.25A_{663} - 2.79A_{645}$$

$$Chlorophyll\ b, C_b\ (mg/L) = 21.50A_{645} - 5.10A_{663}$$

$$Total\ Chlorophyll\ Content, C_{a+b}\ (mg/L) = 7.15A_{663} + 18.71A_{645}$$

Effects of Gamma Irradiation on Rosmarinic Acid Production: Approximately 1.0g of the irradiated and non-irradiated callus was extracted with 10 mL of 99.5% methanol (Mallinckrodt, USA) using mortar and pestle. The extracts were kept in dark and agitated on rotary shaker (PROTECH Model 721, Malaysia) at 80-100 rpm. After 7 days, the extracts were filtered and dried. Approximately 5mL of methanol was then added to the crude extract. Absorbance of the extracts was measured using a spectrophotometer (Bio-Rad, USA) at 327nm. A standard calibration curve of OD_{327} against concentration was constructed using rosmarinic acid (Sigma, USA) at 0-15μg/mL. The amount of rosmarinic acid present in the samples was then further expressed in milligram per gram fresh weight of callus.

Statistical Analysis: Each study was repeated twice with three replicates each and was subjected to statistical analysis. One way ANOVA and Tukey's Honestly Significant Difference test ($p<0.05$) were used to determine the differences between means of all tested parameters between irradiated and non-irradiated callus. Statistical analysis was performed using SPSS software (Release 15.0) (SPSS Inc., USA).

RESULTS AND DISCUSSION

Morphological Studies: The effects of gamma irradiation on the morphology of the callus have been described by many researchers. Investigation on the morphology changes of the irradiated callus allows for the determination of the relationship between callus growth and gamma doses. According to the study conducted by Ahloowalia & Maluszynski [19], the limited number of available reports suggested that callus cultures are much more sensitive to radiation treatment and require much lower doses (2 to 5Gy) than stem cuttings or seeds (15 to 20Gy). The calluses will turn necrotic or lose their regenerative capacity with relatively higher doses (15 to 20Gy). The growth of *Arabidopsis* seedlings exposed to low-dose gamma rays (1 or 2Gy) was slightly increased compared with that of the control, while the seedling growth was noticeably decreased by the high-dose irradiation of 50Gy [20]. Although no conclusive explanations for the stimulatory effects of low-dose gamma radiation are available until now, papers support

a hypothesis that the low dose irradiation will induce the growth stimulation by changing the hormonal signaling network in plant cells or by increasing the antioxidative capacity of the cells to easily overcome daily stress factors such as fluctuations of light intensity and temperature in the growth condition [21].

However, this was not the case for this study as there was no significant difference in the fresh weight of gamma irradiated calluses. Callus exposed to 10Gy showed the highest increase in fresh weight (202.01%) while callus exposed to 20Gy exhibited the lowest increase in fresh weight (141.82%) (Figure 1). Similarly, there were no significant morphological aberrations observed in the phenotype of the irradiated and non-irradiated calli. All calli were found to be of non-embryogenic, friable, with a yellow-brown colouration with a wet, rough crystalline, unorganized appearance. Similar results were reported by Rao [22], whereby low doses (10 to 50Gy) did not affect the growth rate; differences in fresh and dry weights of *Pharbitis nil* being negligible. However, doses above 100Gy caused a steady decline in the growth rate. Likewise, the results in this study were supported by Anonymous [23] who reported that the fresh weight of the callus of Kars and safflower only showed significant difference at 600Gy and above.

Determination of Total Soluble Protein Content: The total soluble protein content of irradiated and non-irradiated callus was determined after three weeks of culture. The results obtained revealed that the increase in gamma irradiation doses caused a reduction in total soluble protein content (Figure 2). All irradiated calluses exhibited a lower amount of total soluble protein as compared to the non-irradiated calluses. Calluses exposed to 10Gy of gamma irradiation exhibited a total soluble protein content of 6.08±1.66 mg/g FW which was 3.57% lower than that of the non-irradiated calluses, 6.31±1.64 mg/g FW. Likewise, calluses irradiated at 20, 30, 40 and 50Gy recorded a gradual decrease in total soluble protein content of 5.74±1.33 mg/g FW (8.99%), 4.98±1.12 mg/g FW (21.10%), 4.84±1.74 mg/g FW (23.18%) and 4.31±1.63 mg/g FW (31.63%) respectively. The effects of gamma irradiation on soluble protein content of bean callus culture was also analysed in an experiment by Bajaj[24]. It was reported that at low doses of irradiation (20 to 30Gy), there was no significant difference in soluble protein content.

Gamma irradiation causes an irreversible change to protein conformations at the molecular level by breakage of covalent bonds of the polypeptide chains [25]. According to Bajaj [24], gamma irradiation causes damage and failure to RNA which causes failure to protein synthesis leading to a decrease in total soluble protein content. 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 DNA transcription and leads to a decrease in protein synthesis and growth.

Determination of Specific Activity of Peroxidase: Peroxidase plays an important role in a variety of cellular functions such as lignification, cell wall biosynthesis and plasticity. Thus, it is important to analyse the changes in peroxidase activity upon exposure to gamma irradiation which may alter cellular functions. In the present study, all irradiated calluses exhibited a lower amount of specific activity of peroxidase as compared to the non-irradiated calluses (Figure 3). Calluses exposed to 10Gy of irradiation showed a slight decrease in specific activity of peroxidase, 1579.75±836.25 units/mg soluble protein which was 4.41% lower than the non-irradiated calluses (1652.68±1160.78 units/mg soluble protein). Calluses subjected to 20, 40 and 50Gy recorded a decrease in peroxidase activity to 1234.73±1134.34 units/mg soluble protein (25.29%), 491.10±416.12 units/mg soluble protein (70.28%) and 522.37±463.56 units/mg soluble protein (68.39%), respectively. Calluses irradiated at 30Gy exhibited peroxidase activity of 347.67±190.58 units/mg soluble protein (78.96%) which was the lowest and the only irradiation which shows significant difference with the non-irradiated calluses according to Tukey's HSD ($p<0.05$).

Gamma irradiation was reported to induce oxidative stress with overproduction of reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\cdot}$), hydroxyl radicals ($OH^{\cdot}$) and hydrogen peroxides (H_2O_2) [28], which react rapidly with almost all structural and functional organic molecules, including proteins, lipids and nucleic acids causing disturbance of cellular metabolism [29]. To avoid oxidative damage, plants have evolved various protective mechanisms to counteract the effects of reactive oxygen species in cellular compartments. In this study, it was discovered that the peroxidase activity of *O. stamineus* callus was in a decreasing trend with higher irradiation doses. This contradicted with the data obtained by Byun *et al.* [30] who showed that gamma irradiation as high as 10kGy, did not induce significant loss in water soluble components such as minerals, nitrogenous constituents, sugars and peroxidase. According to Variyar *et al.* [31], gamma irradiation creates oxidative

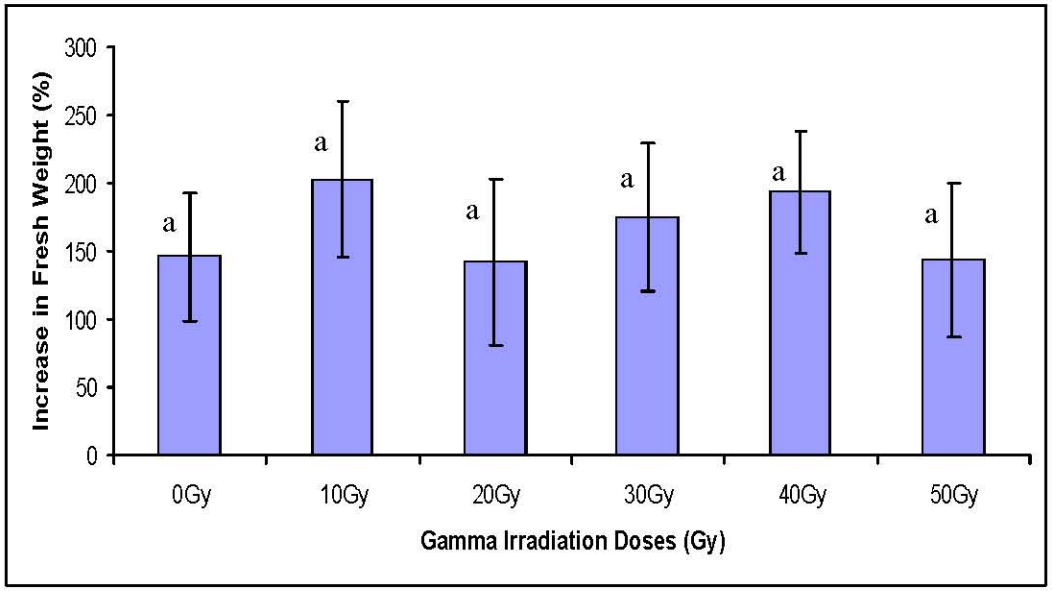

Fig. 1: Effects of gamma irradiation on the increase in fresh weight of *O. stamineus* callus after three weeks of irradiation. Mean with different letter(s) are significantly different between treatments by the Tukey's HSD ($p<0.05$). Error bars indicate the mean ± standard error

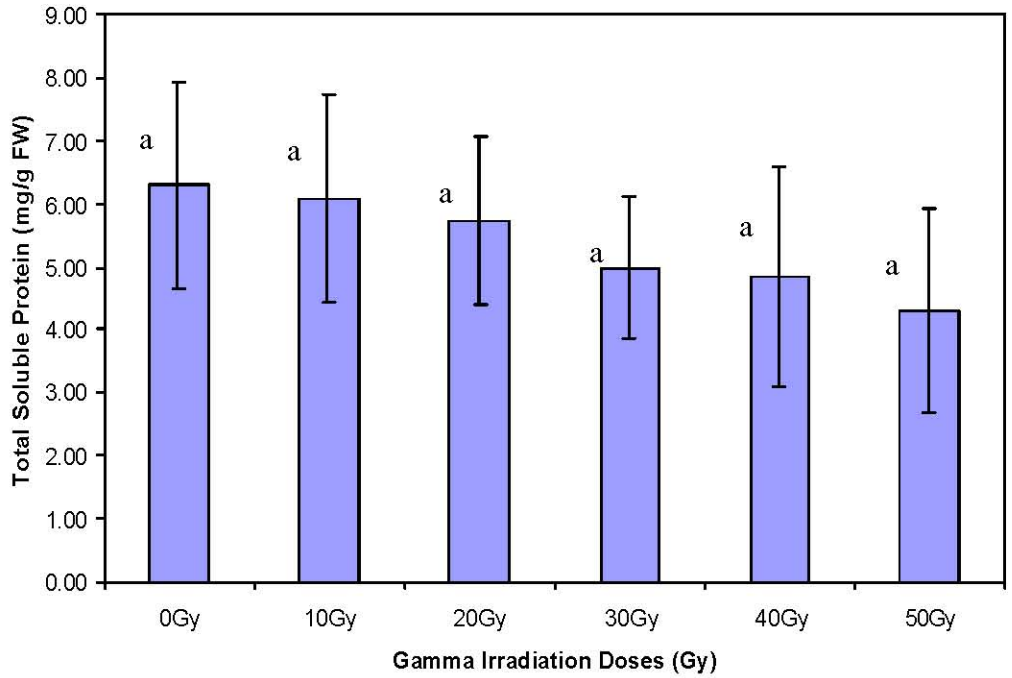

Fig. 2: Effects of gamma irradiation on the total soluble protein content in callus of *O. stamineus* after 3 weeks of irradiation. Mean with different letter(s) are significantly different between treatments by the Tukey's HSD ($p<0.05$). Error bars indicate the mean ± standard error

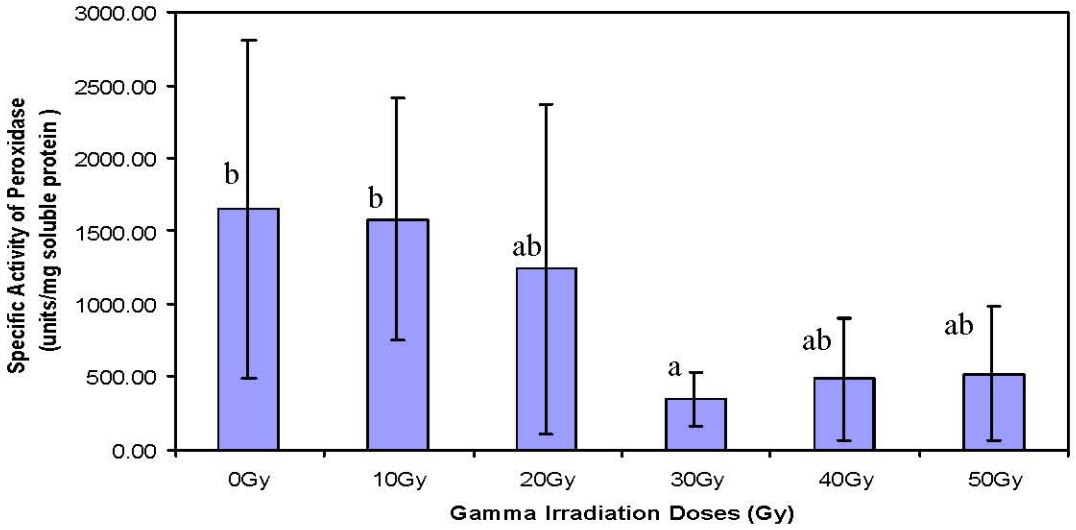

Fig. 3: Effects of gamma irradiation on the specific activity of peroxidase of *O. stamineus* callus after 3 weeks of irradiation. Mean with different letter(s) are significantly different between treatments by the Tukey's HSD ($p<0.05$). Error bars indicate the mean ± standard error

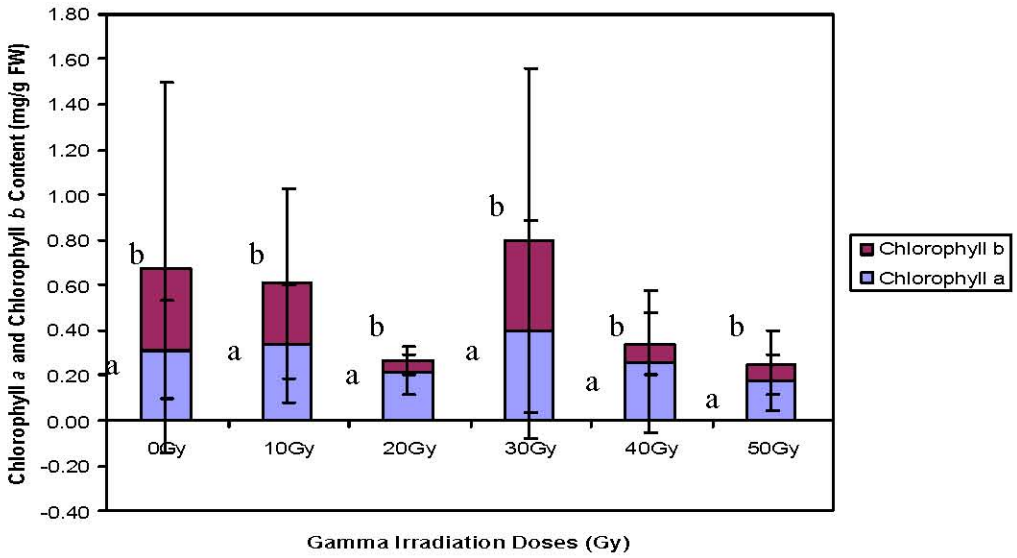

Fig. 4: Effects of gamma irradiation on the chlorophyll *a* and chlorophyll *b* content of *O. stamineus* at 3 weeks of irradiation. Mean with different letter(s) are significantly different between treatments by the Tukey's HSD ($p<0.05$). Error bars indicate the mean ± standard error

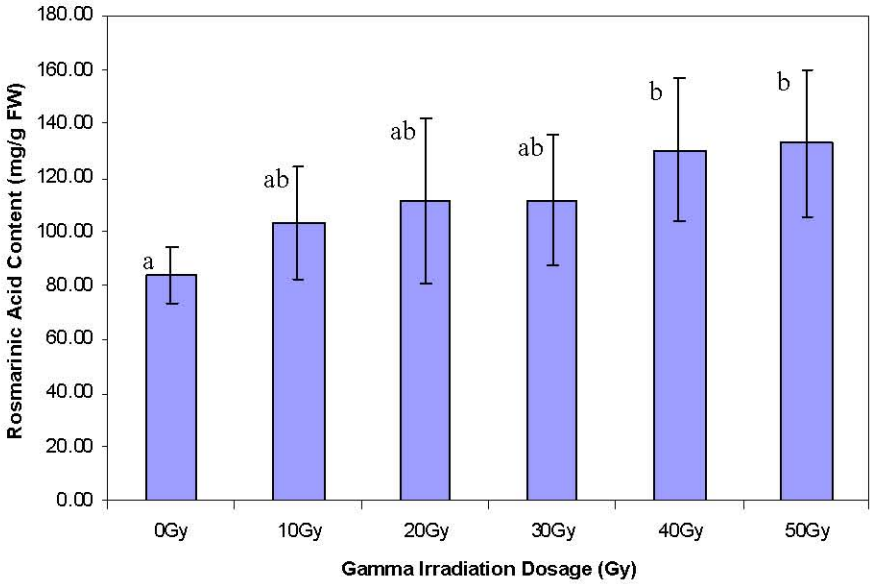

Fig. 5: Effects of gamma irradiation on the rosmarinic acid content of *O. stamineus* at 3 weeks of irradiation. Mean with different letter(s) are significantly different between treatments by the Tukey's HSD ($p<0.05$). Error bars indicate the mean ± standard error

stress and affects biomolecules by causing conformational changes, oxidation, rupture of covalent bonds and formation of free radicals such as hydroxyl and superoxide anion that were generated by radiation. Radiation causes oxidative injury by accelerating free radical generation in living systems. The primary damage induced by ionizing radiation is modified in enzymatic repair processes [32].

Determination of Chlorophyll Content: The chlorophyll content of three-week old irradiated and non-irradiated calluses revealed that exposure to gamma doses caused a reduction in chlorophyll *a* and chlorophyll *b* (Figure 4). Exposure of calluses to 30Gy however displayed an exception where there was increment of chlorophyll *a* and chlorophyll *b* content to 0.76±1.23 mg/g FW which was a 15.62% increase as compared to the non-irradiated calluses (0.66±0.98 mg/g FW). Calluses exposed to 10Gy irradiation exhibited a chlorophyll *a* and chlorophyll *b* content of 0.56±0.67 mg/g FW which was 15.04% lower than that of the non-irradiated calluses. Calluses irradiated at 20, 40 and 50 Gy, 0.23±0.16 mg/g FW (64.66%), 0.22±0.20 mg/g FW (66.26%) and 0.24±0.26 mg/g FW (63.64%) respectively, displayed remarkable decline of more than 60% as compared to the non-irradiated calluses. However, according to Tukey's HSD ($p<0.05$), changes in chlorophyll *a* and chlorophyll *b* content for the calluses were not significantly different from each other. The concentration of chlorophyll *b* (0.36 mg/g FW) was found to be higher than chlorophyll *a* (0.31 mg/g FW) in non-irradiated calluses. However, as illustrated in Figure 4, the concentration of chlorophyll *a* was found to be higher than chlorophyll *b* in irradiated calluses.

In this study, the total chlorophyll content was found to be the highest in the 30Gy irradiated callus. This however contradicted with the studies done by Alikamanoglu *et al.* [32] who reported that a gradual decrease in total chlorophyll content from non-irradiated sample of *P. tomentosa* callus to 25Gy of irradiation. Koh and Davies [33] also claimed that the wild type plant had greater chlorophyll *a*, *b* and total chlorophyll content than the mutant phenotypes. According to Wi *et al.* [20], chloroplasts were extremely sensitive to gamma irradiation as compared to other cell organelles which may give rise to dilation of thylakoid membranes, alteration in photosynthesis, modulation of the antioxidative system and accumulation of phenolic compounds.

Effects of Gamma Irradiation on Rosmarinic Acid Production: Irradiated and non-irradiated calluses were subjected to estimation of rosmarinic acid content after 3 weeks of culture. Figure 5 illustrates that increase in gamma irradiation doses caused an increase of rosmarinic acid content. Calluses irradiated at 40Gy (130.22±26.60 mg/g FW) and 50Gy (132.64±27.03 mg/g FW) showed a sharp increase as compared to the

non-irradiated calluses (83.86±10.60 mg/g FW). According to Tukey's HSD ($p<0.05$), the 55.28% and 58.17% increase of 40 and 50Gy, respectively showed a significant increase of rosmarinic acid concentration. Calluses which were subjected to irradiation at 10, 20 and 30Gy exhibited rosmarinic acid concentration of 103.05±20.89 mg/g FW (22.88%), 111.23±30.75 mg/g FW (32.63%) and 111.54±24.08 mg/g FW (33.01%) respectively, which were all higher than the non-irradiated calluses but there was no significant difference between these gamma irradiation doses according to Tukey's HSD ($p<0.05$).

As mentioned by Berezina and Kaushanskii [34], some physical agents like low doses of gamma irradiation, may induce physiological and biochemical changes. As shown in Figure 5, the accumulation of rosmarinic acid was the highest for the callus irradiated with 50Gy. It was reported by Beaulieu *et al.*[35] that a radiolysis of phenolic acids in an aqueous solution led to their degradation and to a notable hydroxylation. Oufedjikh *et al.* [36] reported that gamma irradiation increases the activity of phenylalanine ammonia lyase, which is responsible for the synthesis of phenolic acids. Variyar *et al.* [31] observed the ability of gamma irradiation in increasing polyphenolic acids in soybeans. This was referred to an irradiation induced breakdown of tannins. Neither qualitative nor major quantitative changes of phenolic acids were, however, observed for example in cinnamon and cardamom [37]. Free radicals generated by plants exposed to irradiation may act as stress signals which triggers stress responses in plants, resulting in an increased phenolic acid synthesis which have notable antioxidative properties [38].

CONCLUSIONS

O. stamineus has a very high value in the commercial and pharmaceutical industry due to its medicinal properties. Future studies on protein identification on gamma irradiated samples as well as gene sequencing in order to identify the specific mutation sites on protein transcription and translation will enable the production of a superior mutant with qualities suitable for commercial use. Further investigation on higher dose of gamma irradiation on both plantlets and callus to compare the profile of biochemical studies as well as production of secondary metabolites is also vital in order to gain better understanding on the effects of irradiation and the possibility of increasing the production of medicinally important metabolites found in *O. stamineus*. Effects of other types of physical irradiation can also be conducted on *O. stamineus* as plants react differently to different means of irradiation.

REFERENCES

1. Food and Agriculture Organization of United Nation (FAO) 1999. Glossary of biotechnology and genetic engineering. FAO Research and Technology, pp: 7.
2. König, A., G. Kleter, W. Hammes, I. Knudsen and H. Kuiper, 2003. Genetically modified crops in the EU: food safety assessment, regulation and public concerns. Project Report. European Commission.
3. Baek, M.H., J.H. Kim, B.Y. Chung, J.S. Kim and I.S. Lee, 2005. Alleviation of salt stress by low dose gamma irradiation in rice. Biologia Plantarum, 49(2): 273-276.
4. Kim, J.H., B.Y. Chung, J.S. Kim and S.G. Wi, 2005. Effects of in Planta gamma-irradiation on growth, photosynthesis and antioxidative capacity of red pepper (*Capsicum annuum* L.) plants. J. Plant Biol., 48(1): 47-56.
5. Chakravarty, B. and S. Sen, 2001. Enhancement of regeneration potential and variability by gamma irradiation in cultured cells of *Scilla indica*. Biologia Plantarum, 44: 189-193.
6. Kim, J.S., Y.K. Lee, H.S. Park, M.H. Back and D.H. Kim, 2000. Influence of low dose gamma radiation on the growth of maize (Zea mays L.) varieties. Korean J. Environ. Agricul., 19: 328-331.
7. Charbaji, T. and I. Nabulsi, 1999. Effect of low doses of gamma irradiation on in vitro growth of grapevine. Plant Cell, Tissue and Organ Culture, 57: 129-132.
8. Kim, J.S., Y.K. Lee, H.S. Song, H.S. Park and J.K. Kim, 1999. Effect of low dose ionizing radiation on the growth and yield of soybean cultivars. Korean J. Environ. Agricul., 18: 66-69.
9. Edahiro, J.I., M. Nakamura, M. Seki and S. Furusaki, 2005. Enhanced accumulation of anthocyanin in cultured strawberry cells by repetitive feeding of L-phenylalanine into the medium. J. Bioscience and Bioengineering, 99: 43-47.
10. Lin, L. and J. Wu, 2002. Enhancement of shikonin production in single- and two- phase suspension cultures of *Lithospermum erythorhizon* cells using low-energy ultrasound. Biotechnol. Bioengineering, 78: 81-88.
11. Yoshikawa, N., H. Fukui and M. Tabata, 1986. Effect of gibberellin A3 on shikonin production in *Lithospermum* callus cultures. Phytochemistry, 25: 621-622.

12. Mohil, B., W. Heller and E. Wellmann, 1985. UV-induced biosynthesis of quercetin 3-O-ß-D-glucuronide in dill cell cultures. Phytochemistry, 24: 465-467.
13. Chan, L.K., 2003. [WWW] http://www.symbiosisonline.com/jun03_invitro.html (21st June 2008).
14. Murashige, T. and F. Skoog, 1962. A Revised Medium For Rapid Growth and Bioassays With Tobacco Tissue Cultures. Plant Physiology, 15(1): 473-479.
15. Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(1): 248-254.
16. Kokkinakis, D.M. and J.L. Brooks, 1979. Tomato peroxidase: Purification, Characterization and Catalytic Properties. Plant Physiology, 63: 93-99.
17. Harborne, J.B., 1973. *Phytochemical methods*, Chapman and Hall Ltd., London, pp: 205-207.
18. Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Enzymology, 148(1): 350-381.
19. Ahloowalia, B.S. and M. Maluszynski, 2001. Induced mutations- a new paradigm in plant breeding. Euphytica, 118: 167-173.
20. Wi, S.G., B.Y. Chung, J.S. Kim, J.H. Kim, M.H. Baek, J.W. Lee and Y.S. Kim, 2006. Effects of gamma irradiation on morphological changes and biological responses in plants. Micron, 38(1): 553-564.
21. Kim, J.H., M.H. Baek, B.Y. Chung, S.G. Wi and J.S. Kim, 2004. Alterations in the photosynthetic pigments and antioxidant machineries of red pepper (*Capsicum annuum* L.) seedlings from gamma-irradiated seeds. J. Plant Biotechnol., 47(2): 314-321.
22. Rao, 1975. The role of gibberellins in the growth of floral organs of Pharbitis nil. Plant Cell Physiology, 14: 91-102.
23. Anonymous 1997. Use of radiation technology database main page. [WWW] http://www.rada.or.jp/database/home4/normal/ht-docs/ member/ synopsis/ 020072.html (10th December 2008).
24. Bajaj, Y.P.S., 1970. Effect of gamma-irradiation on growth, RNA, protein and nitrogen contents of bean callus cultures. J. Bot., 23(1): 231-236.
25. Kume, T. and T. Matsuda, 1995. Changes in structural and antigenic properties of proteins by radiation. Radiation Phys. Chem., 46(1): 225-231.
26. Constantin, M.J. and J.E. Love, 1967. Seedling responses of *Vigna sinensis* (L.) savi to gamma and neutron seed irradiation. Radiation Bot., 7: 497-506.
27. Pollard, E.C. and S.A. Davis, 1964. The action of ionizing radiation on transcription (and translation) in several strains of *Escherichia coli*. Radiation Res., 41(2): 375-399.
28. Xienia, U., G.C. Foote, S. Van, P.N. Devreotes, S. Alexander and H. Alexander, 2000. Differential developmental expression and cell type specificity of dictystelium catalases and their response to oxidative stress and UV light. Biochimica et Biophysica Acta, 149: 295-310.
29. Salter, L. and C.N. Hewitt, 1992. Ozone hydrocarbon interactions in plants. Phytochem., 31(4): 4045-4050.
30. Byun, M., I. Kang and T. Mori, 1996. Effect of ?-irradiation on the water soluble components of soybeans. Radiation Phy. Chem., 47: 155-160.
31. Variyar, P.S., A. Limaye and A. Sharma, 2004. Radiation-induced enhancement of antioxidant contents of soybean. J. Agricul. Food Chem., 52(6): 3385-3388.
32. Alikamanoglu, S., O. Yaycli, C. Atak and A. Rzakoulieva, 2007. Effect of magnetic field and gamma radiation on *Paulowinia tomentosa* tissue culture. Biotechnol., 21(1): 129-134.
33. Koh, Y.C. and F.T. Davies, 2001. Mutagenesis and in vitro culture of *Tillandsia fasciculata* Swartz var. fasciculata (Bromeliaceae). Scientia Horticulturae, 87: 225-240.
34. Berezina, N.M. and D.A. Kaushanskii, 1989. Presowing irradiation of plant seeds. Oxonian Press. New Dehli, India.
35. Beaulieu, M., G. Aprano and M. Lacroix, 2002. Effect of dose rate of gamma irradiation on biochemical quality and browning of mushrooms Agaricus bisporus. Radiation Chem., 63(5): 311-315.
36. Oufedjikh, H., M. Mahrouz, M.J. Amiot and M. Lacroix, 2000. Effect of gamma-irradiation on phenolic compounds and phenylalanine ammonia-lyase activity during storage in relation to peel injury from peel of Citrus clementina hort. Ex. Tanaka. J. Agricul. Food and Chem., 48(2): 559-565.
37. Variyar, P.S., C. Bandyopadhyay and P. Thomas, 1998. Effect of gamma-irradiation on the phenolic acids of some Indian spices. Intl. J. Food Sci. Technol., 33(6): 533-537.
38. Fan, X., P.M.A. Toivonen, K.T. Rajkowski and K.J.B. Sokorai, 2003. Warm water treatment in combination with modified atmosphere packaging reduces undesirable effects of irradiation on the quality of fresh-cut iceberg lettuce. Food Chem., 51(1): 1231-1236.