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Introduction

Gamma rays are often used on plants in developing varieties that are agriculturally and economically important and have high productivity potential (Jain et al., 1998). Gamma rays are also very important in mutation breeding and in in vitro mutagenesis in order to develop required features of plants and increase the genetic variability. The many mutant varieties, which are resistant to diseases, cold, salt and with high quality, have been developed (Jain et al., 1998). Research on the effects of gamma irradiation on food sources and animal cells has been done, with little information available on plant cells and no information to date on medicinal plants (Sanada, 1986). In previous studies, gamma irradiation is known to increase nutritional values of food sources and also enhance and accelerate growth of certain vegetables. Gamma irradiation can be useful for the alteration of one or a few physiological characters (Lapins, 1983). Mutagenesis by means of gamma rays has played an important role in the producing new mutants with improved properties which can produce higher amounts of commercially important metabolites (Sanada, 1986).
Orthosiphon stamineus, a member of the Lamiaceae, is a native plant to tropical Asia. It is a popular medicinal herb in Southeast Asia and is commonly known as “Misai Kucing” in Malaysia and Singapore. The leaves have been introduced to Europe and Japan as a health tea (Lee and Chan, 2004). O. stamineus is used for treating diseases such as rheumatism, diabetes, hypertension, tonsilitis, epilepsy, menstrual disorder, gonorrhea, syphilis, renal calculus, gallstone, urinary lithiasis, edema, eruptive fever, influenza, hepatitis, jaundice and biliary lithiasis. (Awale et al., 2002). However, slow seed germination, limited and delayed rooting of the vegetative cuttings has slowed down the mass propagation of this plant (Awale et al., 2002). These problems, together with the variation of plant growth in the fields, resulted in a limited supply of high quality plant materials that can hardly meet the market demand in the pharmaceutical industries. Studies have also indicated that field-grown plants produced inconsistent bioactive compounds such as rosmarinic acid (Tezuka et al., 2000).

At present, there is a lack of information on the effects of in vitro mutagenesis on O. stamineus. The present study aims to tackle this issue by performing the physiological studies on O. stamineus, after exposure to different dosages of gamma rays. Besides, this study also aim to identify the different protein banding profile of gamma-irradiated O. stamineus as well as to estimate the concentration of rosmarinic acid of gamma-irradiated O. stamineus plantlets.

Materials and methods

Plant Materials

Young shoot tips with approximately 1.0 cm in height were excised from the two-week old in vitro plantlets of O. stamineus that were cultured in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962).

Gamma Irradiation

Gamma irradiation was conducted at Malaysian Nuclear Agency at Bangi, Selangor Malaysia, using Caesium-137 source at a dosage rate of 4.640 kGy/hr. Approximately 1cm-shoot tips of O. stamineus were gamma irradiated after 3 days of culture. Gamma irradiation was performed at 0Gy, 10Gy, 20Gy, 30Gy, 40Gy, 50Gy, 60Gy and 70Gy. After irradiation, the shoot tips were transferred to fresh MS basal medium and were maintained at 25 ± 2 °C with the photoperiod of 16 hours light and 8 hours dark.

Radiation Sensitivity Test

Radiation sensitivity test was performed on O. stamineus according to the survival percentage after exposure to the gamma irradiation. It was used to determine the gamma dosage whereby 50% survival was achieved. The survival percentages of irradiated and non-irradiated plantlets were measured at one week interval for a period of three weeks after gamma irradiation.

Sample Extraction

Irradiated and non-irradiated plantlets were added to a pre-chilled mortar in an ice bath. The plantlets were ground with pestle after adding protein extraction buffer at a ratio of 1.0g of plantlet to 3.0mL of extraction buffer. The extracts were then transferred to 1.5mL Eppendorf tubes to be centrifuged (Heraeus, UK) at 12,000 rpm at 4°C for 20 minutes. The supernatant was removed, placed into a new tube and was used to determine the total soluble protein and peroxidase activity of irradiated and non-irradiated plantlets of O. stamineus.

Determination of Total Soluble Protein

Total soluble protein content of irradiated and non-irradiated plantlets was determined according to the Bradford (1976) method using bovine serum albumin (BSA) (Sigma Aldrich, USA) as a standard. A total of 20µL of sample extracts and 80µL of protein extraction buffer was added to test tubes containing 5mL of protein reagent and subsequently vortexed. About 20µL of double distilled water and 80µL of protein extraction buffer together with 5mL of protein reagent was used as the blank. The absorbance was measured at 595nm using spectrophotometer (Bio-Rad smartspec plus, USA). A concentrated stock solution of bovine serum
Fig. 1: Radiation sensitivity test for *O. stamineus*

albumin (BSA) (1mg/mL) was diluted to 50, 100, 150, 200, 250, 300, and 350μg/mL. A standard calibration curve of OD_{protein} versus concentration of BSA in μg/mL was constructed. The total soluble protein content present in the samples were compared to the standard of BSA and further expressed in milligram per gram fresh weight of plant material.

**Determination of Specific Activity of Peroxidase**

The peroxidase activity was determined spectrophotometrically (Bio-Rad smartspec plus, USA) at 420nm. The initial and the maximum absorbances were obtained for the peroxidation of guaiacol in the presence of hydrogen peroxide. About 2.6mL 0.1M sodium phosphate buffer, 0.3mL 1% (v/v) guaiacol (Fisher, USA), 30% (v/v) hydrogen peroxide (Fisher, USA) together with 50μL protein extraction buffer was used as the blank. For the determination of specific activity of peroxidase of the sample extracts, the above mixture was used except by replacing the protein extraction buffer with 50μL of sample extracts. Peroxidase activity was calculated as specific enzyme activity whereby one unit of enzyme activity is the amount of enzyme used to reduce hydrogen peroxide in a cuvette in one minute per mg of soluble protein.

**Determination of Chlorophyll Content**

Chlorophyll content of irradiated and non-irradiated plantlets was determined according to the Lichtenthaler (1987) method. The irradiated and non-irradiated plantlets were added to a pre-chilled mortar in an ice bath. The plantlets were ground with pestle after adding calcium carbonate (CaCO₃) (Spectrum, CA) at a ratio of 1g of plantlets to 2g of CaCO₃, together with 10mL of 80% (v/v) acetone. The sample extracts were filtered using Whatman no. 1 filter paper and followed by washing with 80% (v/v) acetone. The extraction volume was made up to 50mL with 80% (v/v) acetone. Sample extracts were subjected to spectrophotometric determination (Bio-Rad smartspec plus, USA) of chlorophyll at 646nm and 663nm. The chlorophyll *a* (Cₐ) and chlorophyll *b* (Cₐ) 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*, Cₐ = 12.25(OD₆₄₆) − 2.79(OD₆₆₃)
- Chlorophyll *b*, Cₐ = 21.50(OD₆₄₆) − 5.10(OD₆₆₃)
- Total chlorophyll, Cₐ + Cₐ = 7.15(OD₆₄₆) + 18.71(OD₆₆₃)


**Determination of protein banding profile**

SDS-PAGE was carried out on protein extracts of irradiated and non-irradiated plantlets to determine the molecular weight profile of proteins. SDS-PAGE was performed following the method described by Laemmli (1970). The stacking gel was prepared with 4% monomer and resolving gel with 12% monomer. All the protein extracts were diluted to the same concentration of 5mg/gFW using the protein extraction buffer. The diluted protein extracts were made up to a total volume of 40µL. A total of 40µL of the diluted protein extracts were added to 20µL of 2X sample loading buffer and were boiled for 3 minutes and subsequently cooled rapidly on ice before loading into the gel. Approximately 40µL unstained protein ladder (Fermentas, UK) was used as the molecular weight marker with addition of 20µL of 2X sample loading buffer. Electrophoresis was conducted in a vertical slab gel unit (Mini Protein® 3 Cell, Bio-Rad, USA) equipped with a PAC 300 power supply (Bio-Rad, USA). Gel was run at a constant current of 200V for 60 minutes. The gel was further stained with a 40% (v/v) staining solution for 24 hours and destained with a 40% (v/v) destaining solution for 3 hours.

**Estimation of Rosmarinic Acid Content**

Rosmarinic acid content of irradiated and non-irradiated plantlets was determined after two weeks of irradiation according to the method by Lim et al. (2006). Rosmarinic acid was extracted in 95% (v/v) methanol by grinding the irradiated and non-irradiated plantlets using mortar and pestle. The ground plant materials were soaked in methanol for 1 week and kept agitated on a rotating orbital shaker (Protech, USA) at the speed of 75rpm. Subsequently, the ground plant materials were filtered using the Whatman no. 1 filter paper. The filtrate was concentrated to obtain a crude extract of rosmarinic acid. Approximately 4.0mL of 95% (v/v) methanol was added to the concentrated crude extracts prior to spectrophotometric determination. The production of rosmarinic acid was determined spectrophotometrically (Bio-Rad smartspec plus, USA) at 327 nm. Rosmarinic acid calibration curve was constructed using the rosmarinic acid standard (Sigma Aldrich, USA). A concentrated stock solution of rosmarinic acid (10mg/mL) was diluted to 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2mg/mL using 95% (v/v) methanol. A standard calibration curve of OD_{327nm} versus concentration of rosmarinic acid in mg/mL was constructed. The rosmarinic acid content was then further expressed in milligram per gram fresh weight of plant material.

**Statistical Analysis**

In the present study, three replicates were used and the experiment was repeated twice. The morphological and biochemical changes of irradiated and non-irradiated plantlets of *O. stamineus* were subjected to the statistical analysis. One way ANOVA and Tukey Honestly Significant Difference (Tukey’s HSD) test (p<0.05) were used to determine the differences in mean number of all tested parameters between irradiated and non-irradiated plantlets. Statistical analysis was performed using SPSS software (version 11.5) (SPSS Inc. USA).

**Results and discussion**

**Radiation Sensitivity Test**

The results for radiation sensitivity test based on survival percentage of irradiated and non-irradiated plantlets demonstrated that significant reduction in survival percentage was observed with increasing gamma dosage. For the survival percentage of irradiated plantlets to reach 50%, the gamma dosage administered was 72.5Gy as interpolated from the graph in Figure 1. These results were in accordance with the radiation sensitivity test done by Norfadzrin et al. (2007) whereby increasing gamma dosage also decrease the germination percentage and survival percentage of tomato and okra. All plantlets whether subjected to various dosages of irradiation or non-irradiated, demonstrated 100% survival at the first week of culture. The survival percentage of irradiated plantlets dropped remarkably with increased dosage during the second and third week of culture. However, plantlets irradiated at low dosage, 10Gy, were able to withstand the mutagenic effect of gamma ray and exhibited 100% survival for 3 weeks. The survival percentage of plantlets irradiated at the highest dosage, 70Gy, fell considerably from 100% to 55.5% at the second week of culture to 44.4% at the third week of culture resulting in a total decrement of 55.6%. Similar observations were made for plantlets irradiated at 20 to 60Gy whereby all of them displayed a gradual reduction in survival percentage corresponding to the increased in gamma dosage. Sax (1942) and Lea (1947) have shown that survival of plants to maturity depend on the nature and extent of chromosomal damage. Increasing frequency of
chromosomal damage with increasing dosage may be responsible for less germinability and reduction in plant survival and plant growth.

**Determination of Total Soluble Protein**

Total soluble protein contents of irradiated and non-irradiated plantlets were obtained after 3 weeks of culture. The results obtained revealed that increased gamma dosage caused a reduction in total soluble protein content (Figure 2). All the irradiated plantlets exhibited lower amount of total soluble protein as compared to the non-irradiated plantlets, however there were exceptions for plantlets irradiated at 10 and 20 Gy. Plantlets irradiated at 10 Gy exhibited a total soluble protein content of 39.61±0.66 mg/gFW which was 22.3% higher than that of the non-irradiated plantlets, 32.40±1.85 mg/gFW. Likewise, plantlets irradiated at 20 Gy recorded a total soluble protein content of 34.00±0.85 mg/gFW, which was an excess of 4.9% over the non-irradiated plantlets but was not significantly different according to Tukey HSD (p<0.05). Plantlets irradiated at higher dosages from 30 to 70 Gy revealed a gradual drop in total soluble protein content. The Tukey HSD (p<0.05) test revealed that plantlets irradiated at 50, 60 and 70 Gy recorded total soluble protein content of 11.90±0.27 mg/gFW, 9.21±0.34 mg/gFW and 8.22±0.61 mg/gFW respectively, that were not significantly different from one another. A severe decline was seen in plantlets irradiated at high dosage, 70 Gy, whereby there was a 74.8% decrement in total soluble protein content as compared to the non-irradiated plantlets.

Radiation caused oxidative injury by accelerating free radical generation in living systems. The primary damage induced by ionising radiation is modified in enzymatic repair processes (Alikamanoglu et al., 2007). It was previously shown that gamma irradiation significantly influences the cell metabolism and protein synthesis in plant meristem cells (Casaret, 1968). According to the results obtained in the present study, it was observed that increased gamma dosage caused a reduction of total soluble protein content. However, plants irradiated at relatively low dosage (10 and 20 Gy) displayed a higher total soluble protein content compared to their non-irradiated counterparts. This result demonstrated that there was a direct correlation between gamma dosage and protein content. Gamma irradiation caused inhibition of tissue culture growth along with failure of RNA, and subsequently of protein synthesis (Bajaj, 1970). This accounts for the lower protein concentration in plants irradiated at high dosage (70 Gy).

The most crucial function of plant cell is to respond to gamma stress by developing defense mechanisms. This defense was brought about by alteration in the pattern of gene expression (Corthals et al., 2000). Owing to gene expression altered under gamma stress, qualitative and quantitative changes in total soluble protein contents were obvious (Corthals et al., 2000). These proteins might play a role in signal transduction, antioxidative defense, antifreezing, heat shock, metal binding, antipathogenesis or osmolyte synthesis which were essential to a plant’s function and growth (Zolla et al., 1999).

In a study by Bajaj (1970) which analysed the effect of gamma irradiation on soluble protein content of bean callus culture, it had been reported that at high irradiation dosage (80 Gy), soluble protein content continue to decrease. At low dosages (20 and 30 Gy) however, there was no significant difference in soluble protein content of irradiated and non-irradiated cultures. According to Bajaj (1970), gamma irradiation caused inhibition of tissue culture growth along with failure of RNA and subsequently the failure of protein synthesis. In accordance with the results obtained by Staunher et al. (2007) in the study of soybean seeds, 10 Gy dosage caused a slight increased in total soluble protein content, an increase of 11.0% as compared to the non-irradiated seeds. Data obtained by Cho and Song (2000) showed that gamma irradiation, did not induce significant loss in water soluble components such as total soluble proteins, minerals, nitrogenous constituents, and sugars. Staunher et al. (2007) also revealed that at an increased gamma dosage, the quantity of carbonyl-groups in oxidatively modified proteins significantly increased. Introduction of carbonyl groups into amino acid residues of proteins was a hallmark for oxidative modification due to gamma rays exposure (Staunher et al., 2007).

Gamma radiation creates oxidative stress and affects biomolecules by causing conformational changes, oxidation, rupture of covalent bonds and formation of free radicals such as the hydroxyl and superoxide anion that were generated by radiation (Varyiar et al., 2004). These free radicals could modify the molecular properties of the total soluble proteins causing oxidative modifications of the proteins (Wilkinson and Gould, 1996). The chemical changes caused by gamma irradiation in proteins were fragmentation, cross-linking, aggregation, and oxidation by oxygen radicals generated in the radiolysis of water (Davis and Delsignore, 1987). These changes depend on the chemical nature of the protein, its physical state, and the irradiation condition (Woods and Pickaev, 1994). Especially, the effect of gamma irradiation on protein conformation appears to depend on several factors such as protein concentration, the presence of oxygen, and the quaternary structure of proteins (Garrison, 1987).
In general, radiation causes the irreversible changes of protein conformation at the molecular level by breakage of covalent bonds of polypeptide chains (Kume and Matsuda, 1995). Fragmentation involves reaction of α-carbon radicals with oxygen to form peroxy radicals which decompose to fragment the polypeptide chain at the α-carbon. Hydroxy radical and superoxide anion radical generated by radiation could modify primary structure of proteins, which resulted in changes of molecular weight distribution (Garrison, 1987). Besides fragmentation, aggregation of proteins fragmented is also observed. There have been reports on aggregation and cross-linking of proteins by irradiation (Filali-Mouhim et al., 1997). Covalent cross-linkages are formed between soluble proteins and between peptides and proteins (Garrison, 1987).

**Fig. 2:** Effects of gamma irradiation on total soluble protein of *O. stamineus* at 3 weeks of culture. 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.

**Determination of Specific Activity of Peroxidase**

It is of great importance to analyse the changes in peroxidase activity after gamma irradiation because peroxidase was known to be essential for a variety of cellular functions such as lignification, cell wall biosynthesis and plasticity, which all may be altered upon exposure to gamma irradiation. Extreme increased in peroxidase activity was observed after gamma irradiation especially at high dosages whereby 385.36±15.61U/mg, 537.92±26.41U/mg and 635.37±55.64U/mg peroxidase activity were recorded in plantlets irradiated at 50, 60 and 70Gy respectively (Figure 3). In accordance to Tukey’s HSD (p<0.05), plantlets irradiated at low dosages, 10, 20 and 30Gy, exhibited peroxidase activity of 50.12±3.11U/mg, 75.36±3.98U/mg and 130.98±8.45U/mg respectively which were not significantly different as compared to the non-irradiated plantlets, 57.60±4.54U/mg, according to according to Tukey’s HSD (p<0.05).

Plantlets irradiated at 20 and 30Gy recorded peroxidase activities which were an excess of 30.9% and 127.4% respectively over the non-irradiated plantlets. In contrast, plantlets irradiated at 10Gy showed a slight decrement of peroxidase activity, 13.0%, as compared to the non-irradiated plantlets. Conversely, plantlets subjected to irradiation at higher dosages, 40, 50, 60, and 70Gy, displayed a remarkable enhancement of peroxidase activity whereby 70Gy recorded a peroxidase activity of 635.37±55.64U/mg which was approximately ten times the peroxidase activity of the non-irradiated plantlets. As shown in Figure 3, plantlets subjected to 40 and 50Gy displayed peroxidase activities that were not significantly different from each other. The same observation was made for plantlets subjected to 60 and 70Gy.

Plants often face the challenge of several environmental conditions which include such stressors as drought, salinity, pesticides, low temperature and irradiation, all of which exert adverse effects on plant growth and development (Foyer et al., 1994). Gamma irradiation was reported to induce oxidative stress with
overproduction of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxides, which react rapidly with almost all structural and functional organic molecules, including proteins, lipids and nucleic acids causing disturbance of cellular metabolism (Salter and Hewitt, 1992). To avoid oxidative damage, plants have evolved various protective mechanisms to counteract the effects of reactive oxygen species in cellular compartments (Foyer et al., 1994). One of the protective mechanisms was the enzymatic system, which operate with the sequential and simultaneous actions of a number of enzymes including peroxidase (Kovacs and Keresztes, 2002).

The results of this study revealed that tremendous increased in peroxidase activity was observed in irradiated plants. Peroxidases located in the cytosol, vacuole, and cell walls as well as in extra-cellular spaces use guaiacol as electron donors and utilise hydrogen peroxide in the oxidation of various inorganic and organic substrates (Shah et al., 2001). There was compelling evidence which show that the activities of enzymes involved in reactive oxygen species scavenging were altered by several environmental stresses, including gamma irradiation (Al-Rumaih and Al-Rumiah, 2007). The expression patterns of peroxidase genes exhibited increased transcripts upon gamma irradiation of *O. stamineus* which accounts for the gradual increased in specific activity of peroxidase as the gamma dosage increase. Several reports with other plants provided evidence of enhanced activities of peroxidase by gamma irradiation treatment. In fact, it has been suggested by Al-Rumaih and Al-Rumiah (2007), in the study of the effects of ionising radiation on trigonella species that the activity and isozyme patterns of peroxidase in *Nicotiana debneyi* and *Nicotiana tabacum*, increased in response to gamma irradiation treatment.

Chaomei and Yanlin (1993) also reported an increased in the activity of peroxidase with a corresponding decline in growth of *Triticum aestivum* plants under higher irradiation dosages (20, 40, 60, 80Gy). Apart from that, Singh et al. (1993) also reported 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 10 Gy (Lee et al., 2003). It has also been indicated by Stoeva (2002) that gamma irradiation enhanced peroxidase activity of two *Phaseolus vulgaris* cultivars (Plovdiv 10 and Plovdiv 11).

It was made clear by Zaka et al. (2002) that this over expression probably occurs by an efficient regulatory mechanism, adjusting when necessary enzyme expression by positive regulation of the corresponding genes to provide cells with resistance to gamma rays. The present increase in peroxidase activity was reported to compensate for the progressive drop in catalase activity due to gamma ray exposure. Peroxidase was considered to be the key enzyme for the decomposition of hydrogen peroxide, especially under catalase inactivation (Lee et al., 2003). Pasternak (1987) attributed the increased in peroxidase activity to membrane injury and the resulting shift in cellular calcium levels due to gamma rays.

![Fig. 3: Effects of gamma irradiation on specific activity of peroxidase of *O. stamineus* at 3 weeks of culture. 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.](image)
Determination of Chlorophyll Content

Chlorophyll concentrations of irradiated and non-irradiated plantlets were obtained after 3 weeks of culture. The results obtained revealed that increased gamma dosage caused a reduction of chlorophyll $a$ and $b$ concentrations (Figure 4). According to Tukey's HSD ($p<0.05$), all the irradiated plantlets exhibited lower amount of chlorophyll $a$ and $b$ as compared to the non-irradiated plantlets, $47.64\pm1.54$mg/gFW. Plantlets irradiated at 10, 20 and 30Gy exhibited total chlorophyll concentration of $38.75\pm1.76$mg/gFW, $38.69\pm1.14$mg/gFW and $37.32\pm1.40$mg/gFW respectively but were not significantly different from each other. There was a decrease of 18.5% of total chlorophyll content in plantlets irradiated at 10Gy as compared to the non-irradiated plantlets. Plantlets irradiated at 50 and 60Gy, $30.30\pm1.74$mg/gFW and $28.10\pm1.19$mg/gFW respectively, displayed a further decrement of 36.3% and 41.2% in total chlorophyll content as compared to the non-irradiated plantlets. However, the total chlorophyll content of plantlets irradiated at 50 and 60Gy were not significantly different from each other according to Tukey's HSD ($p<0.05$). A remarkable decline in total chlorophyll content was observed in plantlets irradiated at 70Gy. Plantlets irradiated at 70Gy exhibited a total chlorophyll content of $20.99\pm1.49$mg/gFW which was 55.9% lower as compared to the non-irradiated plantlets.

As illustrated in Figure 4, the concentration of chlorophyll $b$ was higher than chlorophyll $a$ in both irradiated and non-irradiated plantlets. Nonetheless, there was an exception in plantlets irradiated at 70Gy whereby a higher concentration of chlorophyll $a$ was evident, $12.18\pm0.67$mg/gFW, as compared to $8.81\pm0.82$mg/gFW for chlorophyll $b$. Concentration of chlorophyll $a$ was 38.6% higher than that of chlorophyll $b$.

Gamma irradiation induces various physiological and biochemical alterations in plants. The irradiation of plants with high dosages of gamma rays disturbs the hormone balance, leaf gas-exchange, water exchange and enzyme activity (Stoeva, 2002). These effects include changes in the plant cellular structure and metabolism such as dilation of thylakoid membranes, alteration in photosynthesis, modulation of the antioxidative system, and accumulation of phenolic compounds (Wi et al., 2006). Photosynthesis is one of the most studied processes under the effects of gamma irradiation accompanied mainly by growth experiments. Despite the diversity of gamma ray targets in plants, it seems that the photosynthetic apparatus is among the main action sites of gamma rays (Kulandaivelu and Noorudeen, 1983).

Photosynthetic pigments can be destroyed by gamma irradiation, with concomitant loss of photosynthetic capacity (Strid et al., 1990). Chlorophylls and carotenoids may be adversely affected by relatively high dosages of gamma irradiation, with carotenoids generally being less affected than the chlorophylls (Pfundel et al., 1992). However, Kim et al. (2004) contradicted this statement by proposing that, chlorophylls are virtually insensitive to low dosage gamma irradiation whereas the levels of carotenoids decline in a dosage dependent manner at the same degree of irradiation. In this study, the chlorophyll contents of gamma irradiated O. stamineus displayed a gradual decrement as the gamma dosage increased. In addition to that, it can be observed that the concentration of chlorophyll $b$ was relatively higher than chlorophyll $a$ in both irradiated and non-irradiated plants. In previous literatures, it has been reported that gamma irradiation resulted in greater reduction in the amount of chlorophyll $b$ as opposed to chlorophyll $a$ (Strid et al., 1990). This statement contradicts the results of this study. The reduction in chlorophyll $b$ is due to a more selective destruction of chlorophyll $b$ biosynthesis or degradation of chlorophyll $b$ precursors (Marwood and Greenberg, 1996).

In a study of the sensitivity of Nicotiana tabacum seedlings to gamma irradiation by Wada et al. (1998), it was found that chlorophyll content was significantly reduced by gamma irradiation when seedlings were exposed to 20 and 50 Gy. In this study, plants irradiated at 70Gy exhibited a total chlorophyll content which was a decrement of 55.9% as compared to the non-irradiated plants. The negative effect of the gamma rays on the growth limiting factors was mainly related to changes in the chlorophyll content and hence photosynthesis. In irradiation stressed pea seeds (80 and 100 Gy), a 23 and 52% decrease in the chlorophyll content was observed, as compared to the non-irradiated seeds. This could be one of the reasons for the reduced photosynthesis rate (Stoeva, 2002).

In another study by Alikamanoglu et al. (2007), however, an increase in chlorophyll $a$, $b$ and total chlorophyll levels was observed in Paulownia tomentosa plants that were exposed to gamma irradiation. When compared to the non-irradiated plants, the total chlorophyll increased 20.6 % in P. tomentosa plants that were exposed to 80Gy gamma dosage (Alikamanoglu et al., 2007). Furthermore, in the study of the effects of gamma irradiation on red pepper plants by Kim et al. (2004), plants irradiated at 16Gy may have some significant increased in their chlorophyll content that can be correlated with stimulated growth. Modulation in photosynthesis in irradiated plants might partly contributed to increased growth (Kim et al., 2004).

Among the various organelles the chloroplasts appear to be the most sensitive to gamma rays. Gamma rays caused chloroplast to lose its structural integrity. The orderly pattern of grana and stroma thylakoids has been lost, and some of the thylakoids appear slightly dilated (Marwood and Greenberg, 1996). Most of the stroma...
has a crystalline appearance which may have the result of water stress imparted by the gamma irradiation treatment (Strid et al., 1990). Teramura (1986) reported that gamma irradiated plants suffered water stress. However, no evidence of incipient plasmolysis could be detected in any of the cells. It was previously shown that gamma irradiation significantly influences the cell metabolism and mitosis in plant meristem cells (Alikamanoglou et al., 2007). In another study with soybean tissue cultures, the chlorophyll contents of plants regenerated from the explants treated with gamma rays were examined and it was found that it had a positive effect on regeneration in soybean tissue cultures (Atak et al., 2004).

Fig. 4: Effects of gamma irradiation on chlorophyll \( a \) and \( b \) concentration of \textit{O. stamineus} at 3 weeks of culture. 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.

Determination of protein banding profile

Protein extracts of irradiated and non-irradiated plantlets were subjected to analysis by SDS-PAGE. Denatured proteins were fractioned by SDS-PAGE in 12% gel and stained by Coomassie Brilliant Blue. By reference to the molecular weight standard, clearly defined major bands were found at 80, 50, 46, 27, 25, 16, 15, 12 and 10kDa with relative mobility of 0.26, 0.43, 0.46, 0.64, 0.67, 0.83, 0.85, 0.93 and 0.99 respectively. As illustrated in Figure 5, both plantlets irradiated at 0 and 10Gy showed a considerable difference in band intensity as compared to other plantlets irradiated at other dosages. Both exhibited higher band intensities at 50, 46, 27, 25, 16, 15, 12 and 10kDa which corresponded to higher amount of denatured protein at these molecular weights. Corresponding to a molecular weight of 30kDa, an extra band was visible only in plantlets irradiated at 40Gy. Additionally, in plantlets irradiated at 50Gy, it was apparent that two bands at 10 and 12kDa were relatively more intense as compared to those irradiated at 20, 30, 40, 60 and 70Gy. Furthermore, it was evident that plantlets irradiated at 0Gy, 10 and 20Gy showed an extra band at 35kDa. It could be deduced from the SDS-PAGE analysis that the protein profile of irradiated and non-irradiated plantlets varied slightly. However, major bands could still be observed in both irradiated and non-irradiated plantlets although the intensities of the bands may differ according to the gamma dosage.

Protein related functional properties in the cell were influenced by various factors, such as protein denaturation, size, structure and conformation, charge, amino acid composition and amino acid sequence of the protein molecules (Zayas, 1997). Zayas (1997) also demonstrated that the denaturation of globular proteins due to gamma irradiation tends to promote the exposure of previously buried non-polar protein sites, leading to increased hydrophobicity and, subsequently, decreased solubility.

In this present study, SDS-PAGE showed no remarkable difference in the protein banding profile between the irradiated and non-irradiated sample extracts. Regardless of the irradiation dosage, electrophoretic patterns are qualitatively and quantitatively quite similar. It was evident in this study that the major protein bands were
at 80, 50, 46, 27, 25, 16, 15, 12 and 10kDa. Similar results were demonstrated by Basch et al. (1985) in the study of milk proteins whereby the electrophoretic separation produced bands at 94, 67, 57, 43, 30, 18 and 14kDa which represents phosphorylase, bovine serum albumin, ovalbumin, ubiquinone, carbonic anhydrase, lactoglobulin and lactalbumin respectively. In accordance to the results obtained by Abu et al. (2005) in the study of Vigna unguiculata, gamma irradiation caused an alteration in the protein banding profile which was probably due to the fact that irradiation caused an increased in the ratio of exposed hydrophilic to hydrophobic amino residues.

Regarding the radiation damage to proteins, there are two types of damages observed, fragmentation and aggregation (Filali-Mouhim et al., 1997). Gamma irradiation at caused the breakdown of polypeptide chain and formation of small molecular weight molecules (Cho and Song, 2000). Similar results were observed in another study of the effect of gamma irradiation on plant proteins by Le Maire et al. (1990). Usually, breakage of covalent bonds in irradiated proteins is shown as new bands below the major band. Also, proteins irradiated may be converted to higher molecular weight aggregates due to the generation of inter-protein cross-linking reactions, hydrophobic and electrostatic interactions, and the formation of disulfide bonds (Davies and Delsignore, 1987). Any amino acid radical formed within a peptide chain could crosslink with an amino acid radical in another protein. Increases in protein molecular weight due to irradiation may be due in part to cross-linking of polypeptides. Increased molecular weight may have led to increased hydrophobicity via, perhaps, the exposure of more non-polar protein sites (Giulivi et al., 2003).

The formation of the high molecular weight protein aggregates was negligible at low dosages, but increases significantly with increasing dosage. As observed in the present study, the 35kDa band was only present in plantlets irradiated at 0Gy, 10 and 20Gy. This may indicate that 35kDa protein had associated with other proteins leading to the formation of higher molecular weight polymers that probably could not pass through the gradient gel and thus absent in protein extracts of plants irradiated at higher dosages. According to Sharabash et al. (1988) SDS–PAGE profiles of the irradiated wheat plants showed that gamma-irradiation, at low dosages, caused a slight breakdown of the polypeptide chain with a concurrent decrease of a major band intensity under loading of the same amount of the protein.

At high dosage above 100Gy, there were cross-linked products of the degraded protein molecules that could not penetrate the running gel (Sharabash et al., 1988). The degradation of protein could be attributed to the direct effect of radiation since 10% of the radiation energy is absorbed directly by the proteins. In the cases of radiation dosages of 35 and 45Gy, drastically different protein profile was observed (Vu Ković et al., 2005). Vu Ković et al. (2005) indicated that protein fragmentation was more pronounced at the lower radiation dosages up to 100Gy, while protein cross-linking prevailed at the radiation dosages above 150Gy.

![Fig. 5: SDS-PAGE gel stained with Coomassie Brilliant Blue showing the protein banding profile of irradiated and non-irradiated O. stamineus. x indicating an extra band at 35kDa, y indicating an extra band at 30kDa and the circle indicating the presence of highly intense bands at 10 and 12kDa in plantlets irradiated at 50Gy.](image-url)
Fig. 6: Effects of gamma irradiation on the rosmarinic acid concentration in *O. stamineus* at optimum week of production. 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.

*Estimation of Rosmarinic Acid Content*

The production of secondary metabolites through a cell culture technology of renowned medicinal plants has been a challenging subject for many researchers. Rosmarinic acid, a polyphenolic acid, is an important and valuable secondary metabolite produced abundantly in *O. stamineus*. Thus, irradiated and non-irradiated plantlets were subsequently subjected to the estimation of rosmarinic acid content. With reference to Figure 6, plantlets irradiated at 10Gy showed the lowest rosmarinic acid concentration, 5.27±0.35mg/gFW, and was 33.8% lower than that of the non-irradiated plantlets. The highest rosmarinic acid concentration was recorded at 30Gy, 8.40±0.57mg/gFW but according to Tukey’s HSD (p<0.05) it was not significantly different from the non-irradiated plantlets, 8.01±0.48mg/gFW. In addition, rosmarinic acid concentrations of plantlets which were subjected to irradiation at 20, 40, 50 and 70Gy were also not significantly different from the non-irradiated plantlets. According to one way ANOVA (p<0.05), there was a significant difference between the rosmarinic acid content and gamma dosage. However, in this study, it cannot be attributed that high gamma dosage will cause a reduction in rosmarinic acid content due to the irregular trend of rosmarinic acid concentration at different gamma dosages as illustrated in Figure 11. In general, plantlets irradiated at 10, 20, 40, 50, 60 and 70Gy exhibited rosmarinic acid concentration of 5.27±0.35mg/gFW, 7.28±0.59mg/gFW, 6.37±0.49mg/gFW, 5.96±0.48mg/gFW, 5.37±0.56mg/gFW and 6.14±0.54mg/gFW respectively, which were all lower than the non-irradiated plantlets.

In the present study, the highest rosmarinic acid concentration was recorded at 30Gy. Thus, it cannot be attributed that rosmarinic acid concentration increased with increasing gamma dosage. This result is contradicted with the results obtained form Luis et al. (2007) in the study of gamma irradiation effects on foliar concentrations of rosmarinic acid in rosemary plants whereby gamma rays significantly increased the concentrations of both rosmarinic and carnosic acids, as well as other rosemary compounds, such as naringin and carnosol. In addition, there have been several reports on gamma rays promotion of polyphenolic acids production, particularly in members of the Lamiaceae family (Johnson et al., 1999).

The ability of gamma irradiation to increase polyphenolic acids in plant material has also been observed in soybeans. Soybean samples treated with gamma irradiation at levels ranging from 50 to 150Gy had increased free polyphenolic acids (Variyar et al., 2004). Siddhuraju et al. (2002) attributed such increase in polyphenolic acids to higher extractability by depolymerization and dissolution of cell wall polysaccharides due to gamma irradiation. Moreover, gamma irradiation was known to increase the activity of phenylalanine ammonia lyase, which is responsible for the synthesis of polyphenolic acids (Oufedjik et al., 2000).
In addition, Song et al. (2002) reported that the total polyphenolic acid contents of the vegetable juice were significantly higher in the irradiated samples than in the non-irradiated samples. Apart from that, in previous works by Beaulieu et al. (2002), it was reported that a radiolysis of polyphenolic acids in an aqueous solution led to their efficient degradation and to a notable hydroxylation. Fan et al. (2003) reported that the free radicals generated in plants during irradiation may act as stress signals and may trigger stress responses in plants, resulting in increased polyphenolic acid synthesis which had notable antioxidative properties.

According to the results of this study, it could be observed that plants irradiated at 10Gy exhibited a significant decrease in rosmarinic acid concentration. Likewise, these results were supported by Urbain (1996) in the study of gamma irradiation of kale plants. In the analysis performed immediately after gamma irradiation, the level of the polyphenolic acids in the irradiated kale plant was significantly lower than the non-irradiated plants. However, the polyphenolic acids of the irradiated kale plants increased after 3 days and soon became higher than the non-irradiated plants. This phenomenon was probably due to the immediate oxidation of polyphenolic acids and thus playing an antioxidative role by reducing the free radicals and the reactive oxygen species induced by gamma irradiation (Urbain, 1996).

**Conclusion**

*O. stamineus* has a promising future for novel discoveries in the commercial and pharmaceutical industry. *In vitro* propagation of *O. stamineus* enables the mass extraction of economically valuable secondary metabolites such as rosmarinic acid which possessed important medicinal properties and is widely used as a food supplement in many countries. Further investigation on the effects of gamma irradiation on *O. stamineus* could be carried out to enable the production of a mutant with superior qualities for commercial use. It is proved to be encouraging for future research to screen for a mutant which is able to produce vast amounts of medicinally important metabolites.

Besides that, ultrastructure studies on gamma irradiated *O. stamineus* could be carried out on important organelles such as mitochondrion, chloroplast and nucleus by utilising scanning electron microscope and transmission electron microscope. Since there was compelling evidence that gamma irradiation significantly caused the alteration in photosynthetic activity, a study on photosynthesis and leaf gas exchange could be carried out in further experiments. In addition, analysis on starch content and total soluble nitrogen could also be done in the future. Seeing that gamma ray is an effective mutagenic agent, it would be useful to instigate a study at the gene level to analyse the modification of certain genes which codes for important cellular products. At the molecular level, agarose gel electrophoresis on DNA and RNA could be performed on gamma irradiated *O. stamineus* to see the difference in gene expression pattern of this plant species.

**Abbreviations**

2,4-D, 2,4-dichlorophenoxyacetic acid; Picloram, 4-amino-3,5,6-trichloropicolinic acid;

**References**


