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Morphological and biochemical responses of *Oryza sativa* L. (cultivar MR219) to ion beam irradiation*

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Abstract: Objective: Heavy ion beam, which has emerged as a new mutagen in the mutation breeding of crops and ornamental plants, is expected to result in the induction of novel mutations. This study investigates the morphological and biochemical responses of *Oryza sativa* toward different doses of carbon ion beam irradiation. Methods: In this study, the dry seeds of *O. sativa* were irradiated at 0, 20, 40, 60, 80, 100, and 120 Gy, followed by in-vitro germination under controlled conditions. Morphological and biochemical studies were conducted to investigate the morphological and physiological responses of *O. sativa* towards ion beam irradiation. Results: The study demonstrated that low doses (10 Gy) of ion beam have a stimulating effect on the height, root length, and fresh weight of the plantlets but not on the number of leaves. Meanwhile, doses higher than 10 Gy caused reductions in all the morphological parameters studied as compared to the control samples. The highest total soluble protein content [(2.11±0.47) mg/g FW] was observed in plantlets irradiated at 20 Gy. All irradiated plantlets were found to have 0.85% to 58.32% higher specific activity of peroxidase as compared to the control samples. The present study also revealed that low doses of ion beam (10 and 20 Gy) had negligible effect on the total chlorophyll content of *O. sativa* plantlets while 40 Gy had a stimulating effect on the chlorophyll content. Plantlets irradiated between 40 to 120 Gy were shown to be 0.38% to 9.98% higher in total soluble nitrogen content which, however, was not significantly different from the control samples. Conclusions: Carbon ion beam irradiation administered at low to moderate doses of 10 to 40 Gy may induce *O. sativa* mutants with superior characteristics.

Key words: In vitro mutagenesis, Ion beam irradiation, Total chlorophyll content, Total soluble protein content, Mutation breeding

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1 Introduction

Mutation techniques have been used for generating vast genetic variations and breeding thousands of new crop varieties during the past decades (Fu *et al*., 2008). According to Sharma (1986), the crop plant characteristics that have been improved by mutation breeding are generally those that have either not found favor with natural selection or were not achieved during previous plant breeding efforts. The successful achievement with mutation breeding techniques for the improvement of the major crops in the

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world would indicate that it is no longer a controversial breeding technique, but an important way to complement the conventional breeding technology (International Rice Research Institute, 1985). Recently, heavy ion beam irradiation has been established as an effective method of inducing mutations in many plant species due to its high frequency and broad spectrum (Hayashi *et al*., 2007). Ion beam irradiation is expected to result in different mutation induction effects and induction of novel mutations that have not been previously studied (Yamaguchi *et al*., 2009).

Mutation breeding of rice has been very successful. Induced mutagenesis in rice has dated back to as early as the 1930s, while the first radiation-induced mutant variety of rice, Reimi, was released in 1966 in Japan as a national registered variety (Sharma, 1986). Induced mutagenesis plays a vital role for the improvement of rice by developing a large number of semi-dwarf and high-yielding varieties around the world (Balooch *et al*., 2006). According to Wang (2006), attempts to improve the traits of rice to overcome the major stresses through conventional breeding techniques have had limited success. In this case, mutation breeding is a good way to complement conventional breeding since it is very effective in improving the agronomic traits, resistance to stress, grain physical parameters, and nutritional value of the rice (Mohamad *et al*., 2006).

The combination of mutation breeding and plant cell and tissue culture techniques made possible effective screening and selection of desired mutants followed by subsequent propagation, maintenance, and manipulation of plant germplasm (Suprasanna *et al*., 2009). The use of an in vitro system for selection of mutants is advantageous as millions of cells can be screened rapidly in a small space, under precisely controlled conditions (Widholm, 1989). As such, the current study was conducted with the aim of determining the morphological and physiological responses of *Oryza sativa* MR219 toward ion beam irradiation at different doses. *O. sativa* MR219 was used as it is a new rice variety developed in Malaysia and possesses several improved traits such as a maturation period between 105 and 111 d, large grain size, and good eating quality. Thus, this study attempted to further improve the quality and traits of MR219 through ion beam irradiation.

2 Materials and methods

2.1 Plant materials and irradiation method

The ion beam-irradiated seeds of *Oryza sativa* L. (cultivar MR219) were obtained from the Malaysian Nuclear Agency. About 150 dry rice seeds were placed in upward embryo on a petri dish and subjected to 320 MeV carbon ion irradiation at 0, 10, 20, 40, 60, 80, 100, and 120 Gy, respectively, by a vertical beam line of AVF cyclotron in JAERI, Takasaki, Japan. The irradiation was performed under atmospheric pressure within 3 min.

2.2 Preparation of the culture medium

MS medium (Murashige and Skoog, 1962) was used as the basal culture medium for seed germination in this study. A total of 3% of sucrose (0.03 g/ml; Sigma Aldrich, USA) was added as the carbon source. The pH of the medium was adjusted to 5.7±0.1 with 0.1 mol/L sodium hydroxide (Sigma Aldrich, USA) or 0.1 mol/L hydrochloric acid (Sigma Aldrich, USA). Plain agar at 0.8% (8 g/L) was added to the culture medium followed by heating in the microwave oven for 5 min to dissolve the agar. Subsequently, approximately 12.5 ml of medium was poured into each of the culture vials (25 mm×100 mm) and allowed to cool and the vials were carefully covered with aluminium foil prior to autoclaving at 121 °C and 15 psi (1 psi=6.895 kPa) for 15 min.

2.3 Surface sterilization and germination of seeds

The seeds that were irradiated at different doses were soaked in 75% ethanol (R&M, UK) for 5 min to eliminate contaminants on the surface of the seed coats. The seed coats were then carefully removed prior to washing under running tap water for 30 min. Surface sterilization was initiated by immersing the seeds in 25% Clorox® solution containing three drops of Tween-20 (Amresco, USA) for 10 min, coupled with continuous shaking. Subsequently, the seeds were rinsed with sterile distilled water for 5, 10, and 15 min, respectively, to eliminate the traces of Clorox®. The surface-sterilized seeds were then cultured on a basal MS medium to allow further germination.

2.4 Culture conditions

All the cultures in this study were maintained for 14 d prior to morphological and biochemical studies,

under controlled conditions of (25±2) °C, with a photoperiod of 16 h light and 8 h dark.

2.5 Morphological studies

Both control and irradiated plantlets of *O. sativa* were subjected to morphological studies, whereby, the germination percentage of the seeds and the morphological appearance of the plantlets such as height, number of leaves, length of roots, and fresh weight (FW) were determined. The germination percentage of the cultured seeds was determined in Week 1 of the culture. Meanwhile, the height, number of leaves, length of roots, and FW of the plantlets were observed and recorded in Week 2 of the culture. The length of the roots was measured using a ruler based on the longest root of each plantlet.

2.6 Sample extraction

Plantlets of irradiated and non-irradiated seeds were extracted with Tris protein extraction buffer (pH 8.0) at the ratio of 1 g of sample to 10 ml of protein extraction buffer. The samples were homogenized in an ice bath with the addition of a protein extraction buffer. The crude extracts were then transferred to a 15 ml centrifuge tube followed by centrifugation at 12000 r/min for 20 min at 4 °C. The resulting supernatant was transferred to a new centrifuge tube and was used for the determination of total soluble protein content and specific activity of the peroxidase of the *O. sativa* plantlets.

2.7 Determination of total soluble protein content

The total soluble protein contents of the irradiated and non-irradiated *O. sativa* plantlets were determined by using the method of Bradford (1976). The total soluble protein assay was carried out by addition of 20 μl of sample extract to 80 μl of protein extraction buffer and 5 ml of protein reagent. The solution was thoroughly mixed by vortexing, followed by a determination of absorbance at 595 nm using GENESYS 20 spectrophotometer (Thermo Scientific, USA). The total soluble protein content of the samples was determined from the standard curve plotted by using bovine serum albumin (BSA; Sigma Aldrich, USA) as the standard at 0, 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 mg/ml. The total soluble protein content of the samples was then expressed in mg/g FW of the plant material.

2.8 Determination of specific activity of peroxidase

The specific activities of peroxidases of irradiated and non-irradiated plant samples were determined by the method of Kokkinakis and Brooks (1979). The oxidation of guaiacol by peroxidases in the presence of hydrogen peroxide (H_2O_2) was used as the basis for the colorimetric assay based on the development of a reddish color (Doerge *et al*., 1997). The assay was carried out by adding 1.0 ml of 30% H_2O_2 (R&M, UK) to the reaction mixture consisting of 0.5 ml of sample extracts, 7.5 ml of 0.1 mol/L sodium phosphate buffer at pH 6.1, and 1.0 ml of 1% guaiacol (0.01 g/ml; Merck, Germany). The solution was quickly mixed by vigorous shaking, followed by monitoring of the absorbance changes at 420 nm for 3 min. The initial absorbance reading at 0 min and the maximum absorbance reading within 3 min were recorded. The unit of specific peroxidase activity was expressed in U/mg soluble protein, in which one unit of activity is defined as the amount of enzymes that causes a change in absorbance by 0.01 per minute.

2.9 Determination of chlorophyll content

The chlorophyll content of the plantlets was determined by the method of Lichtenthaler (1987). Chlorophyll was extracted by grinding the plant samples in an ice bath with 10 ml of 80% acetone at the ratio of 1 g sample to 2 g of calcium carbonate ($CaCO_3$). The extract was filtered and collected in a measuring cylinder. The extraction volume was brought up to 50 ml with 80% acetone before the absorbance of the sample extract was determined at 645 and 663 nm, respectively, using the GENESYS 20 spectrophotometer (Thermo Scientific, USA). The chlorophyll a (C_a) and chlorophyll b (C_b) contents of the samples in mg/L were then determined. The chlorophyll content in mg/L was further expressed as mg/g FW of the plant material.

2.10 Determination of total soluble nitrogen content

Plant samples were extracted with 20 ml of 80% ethanol by grinding in an ice bath. The crude extracts were then transferred to 50 ml centrifuge tubes and were centrifuged at 14000 r/min for 5 min under room temperature. The resulting supernatant was removed and transferred into boiling tubes followed by the addition of ninhydrin solution at a ratio of 0.2 ml of

ninhydrin solution to 20 ml of crude extracts. The boiling tubes were then covered with aluminium foil and vortexed prior to heating in a boiling water bath. The samples were heated in a boiling water bath for approximately 20 min until 5 ml of extracts were left as the final volume. Subsequently, 5 ml of diluent was added to the sample extracts before the absorbance was measured at 570 nm using a GENESYS 20 spectrophotometer (Thermo Scientific, USA). The total soluble nitrogen content of the samples was determined from the standard curve plotted by using leucine as a standard at concentrations of 0, 0.003, 0.005, 0.008, 0.011, 0.013, 0.016, 0.018, 0.021, 0.024, 0.026, 0.029, and 0.032 mg/ml and was further expressed in mg/g FW of the plant material.

2.11 Statistical analysis

In this study, the experiments were carried out in ten replicates for both morphological and biochemical studies where each set of the experiments was repeated once. The results obtained from the morphological and biochemical studies of the plant samples were subjected to one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test at $P<0.05$ to determine the significance differences between the mean of each parameter tested in the study. These statistical analyses were performed by using SPSS software (Version 15.0, SPSS Inc., USA).

3 Results and discussion

3.1 Height of plantlets

The morphological study was carried out after two weeks of culture to compare the heights of the irradiated and non-irradiated plantlets (Fig. 1). In general, similar height was observed on both irradiated and non-irradiated plantlets after two weeks of culture on a basal MS medium. According to Tukey's HSD test ($P<0.05$), there was no significant difference in the height between all the irradiation doses. However, all the plantlets of the irradiated seeds showed a slight reduction in their height as compared to the control samples, with an exception for plantlets irradiated at 10 Gy of carbon ion beam, which were 3.9% taller than the control samples.

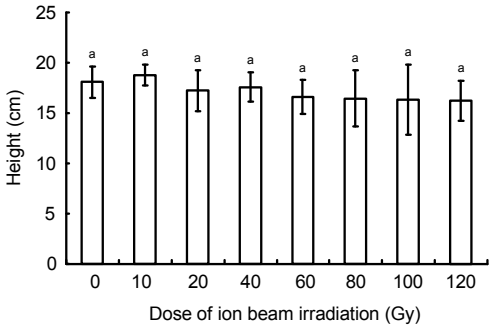

Fig. 1 Effects of carbon ion beam irradiation on the height of *O. sativa* after two weeks of culture
Means with different letters are significantly different between treatments by the Tukey's HSD test ($P<0.05$). Error bars indicate the mean±standard deviation ($n=10$)

The present study revealed that inhibited growth in terms of height was evident in *O. sativa* plantlets with increasing doses of carbon ion beam administered on the seeds. Similar results were reported by Masuda *et al*. (2009) who noticed a reduction in radical elongation as a result of irradiation of tomato seeds by carbon and helium ions. The negative impact of radiation on plants may be indirectly mediated via metabolic changes through free radical formation, as well as indirectly, by DNA damage to the dividing cells (Jones *et al*., 2004). According to Vazquez-Tello *et al*. (2005), cell division is the most sensitive parameter to irradiation, which might account for the growth inhibition observed in irradiated plantlets. High doses of irradiation may cause cell cycle arrest at the G_2/M phase during somatic cell division or damaging of the genome, leading to growth inhibition (Preuss and Britt, 2003).

On the contrary, stimulated growth was observed on *O. sativa* plantlets derived from seeds that were irradiated with low doses of carbon ion beam. The stimulation of plant growth at low irradiation doses was frequently observed on γ-irradiated plant materials (Kim *et al*., 2004; Ling *et al*., 2008). This may be due to the hormetic effects of low-dose ionizing radiation on plants that are manifested as accelerated cell proliferation, stimulated germination and growth, improved stress-resistance, or even increased yield (Kim *et al*., 2004). In many cases, such effects could be characterized as the modulation of photosynthesis and antioxidant machineries (Kim *et al*., 2004).

3.2 Length of roots

The length of roots of irradiated and non-irradiated plantlets was also used as a parameter to investigate the morphological responses of *O. sativa* towards different doses of carbon ion beam irradiation. The root length of the plantlets was measured in Week 2 of the culture. The root of plantlets irradiated at 10 Gy was 0.41% longer than that of the control, while plantlets irradiated at 20, 40, 60, 80, 100, and 120 Gy were found to have relatively short roots as compared to the control samples (Fig. 2). This revealed that ion beam irradiation at high doses resulted in plantlets with relatively short roots. Although there was an overall decrease in the root length with the increase of ion beam doses (except 10 Gy), statistical analysis revealed that there was no significant difference between all the treatments.

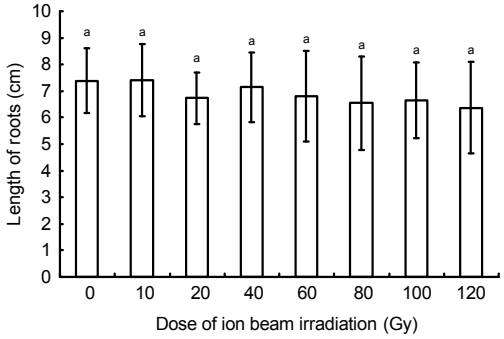

Fig. 2 Effects of carbon ion beam irradiation on the root length of *O. sativa* after two weeks of culture
Means with different letters are significantly different between treatments by the Tukey's HSD test (P<0.05). Error bars indicate the mean±standard deviation (n=10)

In a previous study conducted by Tanaka *et al*. (2002) where root apical tissues of *Arabidopsis* were irradiated with 220 MeV carbon ion beam, it was discovered that the vertical root elongation showed a small increase up to about 10 Gy, followed by an exponential decrease until 75 Gy. Meanwhile, root growth was completely inhibited at 100 Gy of carbon ion beam irradiation (Tanaka *et al*., 2002). These findings were similar to the results observed in the present study, whereby the plantlets exhibited better rooting ability at low doses of carbon ion beam irradiation followed by a gradual decrease in root length with increasing doses of carbon ion beam. This was also in accordance with the findings of Rakwal *et al*. (2007), who discovered that the root growth of a rice plant was reduced in a dose-dependent manner 3 d after irradiation with a carbon ion beam. Another study by Kalimullah *et al*. (2003) suggested that the elongation of root in *O. sativa* plantlets derived from ^{1}H heavy ion irradiated seeds was about 17% less as compared to the control samples. Although there was a constant increase in the length of roots observed in irradiated samples, it was always less than that of the control samples, indicating that irradiation affected the growth of roots (Kalimullah *et al*., 2003). Skoog (1935) and Gordon and Weber (1953) reported that the destruction and inactivation of endogenous auxin balance by irradiation might be accounted for by the reduced rooting ability of the treated plants.

3.3 Number of leaves

In this study, the number of leaves formed on *O. sativa* plantlets was recorded after two weeks of culture. From the results obtained, 10 Gy carbon ion irradiated plantlets were found to have the same leaf number [(2.9±0.7) leaves] with non-irradiated plantlets. Meanwhile, all the plantlets irradiated at 20, 40, 60, 80, 100, and 120 Gy were found to have smaller leaf numbers as compared to the control samples (Fig. 3). However, statistical evidence showed that the number of leaves of the plantlets was not significantly different between all the treatments, indicating that all doses of carbon ion beam irradiation have similar effects on the leaf number of *O. sativa* plantlets.

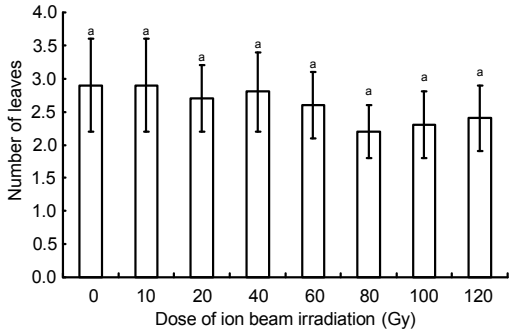

Fig. 3 Effects of carbon ion beam irradiation on the number of leaves of *O. sativa* after two weeks of culture
Means with different letters are significantly different between treatments by the Tukey's HSD test (P<0.05). Error bars indicate the mean±standard deviation (n=10)

As demonstrated by Ahmad *et al*. (2006), protocorm-like bodies that were irradiated with 220 MeV carbon ions at 12 Gy did not manage to

elongate into complete shoots. Another study by Matsumura *et al*. (2010) showed that the frequencies of shoot primordial formation on ray floret explants of chrysanthemum as well as the shoot bud formation on leaf explants of chrysanthemum cultivar H13 were decreased by 8 Gy and 4 Gy of carbon ion beam irradiation, respectively. Meanwhile, Hewawasam *et al*. (2004) noticed a reduction in the mean number of secondary shoots produced per culture with increasing doses of irradiation. These studies suggested that carbon ion beam irradiation may cause considerable inhibition in leaf or shoot formation even at relatively low doses. According to Hewawasam *et al*. (2004), the abnormal development of leaves in the treated population was probably due to physiological disturbance and chromosome aberration as a result of irradiation.

3.4 Fresh weight

Apart from height, root length, and leaf number, the FW of *O. sativa* plantlets was also used as a means to determine the effect of carbon ion beam irradiation on the growth of *O. sativa*. In general, all irradiated plantlets demonstrated a lower FW as compared to the control samples, with the exception of plantlets irradiated with 10 Gy of carbon ion beam. The FW of plantlets irradiated at 10 Gy was (0.1221±0.0219) g, which was 5.62% greater than that of non-irradiated plantlets (Fig. 4). On the other hand, plantlets irradiated at 60 Gy exhibited the lowest FW among all the treatments, which was (0.0963±0.0164) g. Statistical evidence based on Tukey's HSD test (P<0.05) showed that the FW of the plantlets was not significantly different between all the treatments.

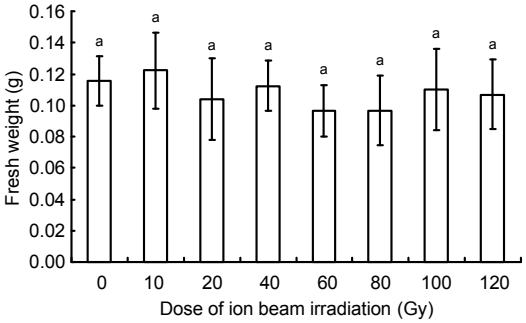

Fig. 4 Effects of carbon ion beam irradiation on the fresh weight of *O. sativa* after two weeks of culture
Means with different letters are significantly different between treatments by the Tukey's HSD test (P<0.05). Error bars indicate the mean±standard deviation (n=10)

Similarly, present investigation was consistent with the findings of Zhou *et al*. (2006), whereby the rate of increase in the FW of irradiated in vitro explants of *Saintpaulia ionahta* was found to decrease with increasing dose of ion beam. This suggests that ion beam irradiation had inhibited the growth of leaf explants. Similar findings were reported by Dong *et al*. (2008) who observed the effect of irradiation as inhibition of seedling growth of sweet sorghum, which directly affected its FW. The effect of radiation on FW of plants might be due to the reduced amount of endogenous growth regulators, especially cytokines, as a result of the breakdown or lack of synthesis due to irradiation (Omar *et al*., 1993).

3.5 Total soluble protein content

The biochemical assay revealed that the total soluble protein content of *O. sativa* plantlets showed some variability with different doses of carbon ion irradiation. According to Fig. 5, irradiation of seeds at lower doses (10 to 40 Gy) resulted in plantlets with relatively high total soluble protein content compared to the control samples. However, irradiation with higher doses (60 to 120 Gy) of carbon ion beam gradually reduced the total soluble protein content of the resulting *O. sativa* plantlets.

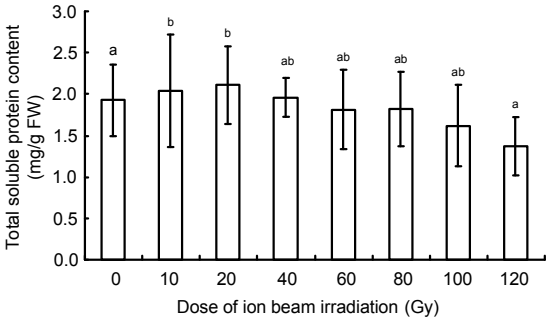

Fig. 5 Effects of carbon ion beam irradiation on the total soluble protein content of *O. sativa* after two weeks of culture
Means with different letters are significantly different between treatments by the Tukey's HSD test (P<0.05). Error bars indicate the mean±standard deviation (n=10)

Ion beam irradiation on the seeds of *O. sativa* causes a considerable stress on the plant. Plants that are sessile and unable to escape radiation exposure in their environment (Rakwal *et al*., 2007) often develop unique molecular mechanisms to cope with different

stress factors (Rao, 2006). Plants may alter their physiologies, metabolic mechanisms, gene expressions, and developmental activities to cope with the stress effect (Rao, 2006). The stress reaction of plants often results in an alteration of gene products (Humera, 2006), which plays a key role in the molecular mechanisms of stress tolerance (Rao, 2006).

Cho *et al*. (2000) demonstrated that various genes displayed differential patterns of gene expression in response to irradiation in tobacco plants, thereby suggesting that a complex signaling mechanism is involved in the irradiation-induced defense by plants. As a result of altered gene expression under radiation stress, the qualitative and quantitative changes in protein content were obvious (Corthals *et al*., 2000). These proteins might play a role in signal transduction, anti-oxidative defense, anti-freezing, heat shock, metal binding, anti-pathogenesis, and osmolyte synthesis, which are essential to a plant's function and growth (Qureshi *et al*., 2007). This might be the possible explanation for the increase in total soluble protein content at low or mild irradiation doses as observed in this study.

On the other hand, Hayden and Friedberg (1964) mentioned that radiation may disrupt hydrogen bond, destroy sulfydryl groups, or affect other reactive groups in the side chain of protein molecules. This can cause irreversible change in the protein conformation, which tends to occur with high irradiation doses. In addition, fragmentation and aggregation of protein molecules might also occur in response to radiation exposure (Gaber, 2005). Apart from that, high linear energy transfer (LET) radiation, such as ion beam, is able to produce double-strand breaks in the DNA with low reparability (Shikazono *et al*., 2003), directly affecting the gene expression and protein synthesis mechanism. These phenomena might lead to a reduction in total soluble protein content when the seeds were irradiated at high doses of ion beam, as observed in the current study.

Extensive research has been carried out to investigate the effects of γ irradiation and other ionizing radiation on the total protein content of plants, while little research evidence was found regarding the effects of ion beam irradiation on the protein content in a plant model. In the study of Maity *et al*. (2009), which investigated the effect of γ irradiation on edible seed protein, it was discovered that the total protein content decreased with increasing doses of γ irradiation as compared to the control samples. Bajaj (1970) showed that soluble protein content of bean callus gradually decreased with the increase in the dosimetry of γ irradiation. In contrast, Iqbal *et al*. (1974) revealed that there was a significant increase in protein contents of corn seeds and *Zea mays* after irradiating the seeds at 0, 17.39, 86.96, 130.40, and 173.90 Gy of γ rays. Similarly, Khanna and Maherchandani (1985) discovered an increase in the protein content of *O. sativa* after irradiation.

3.6 Specific activity of peroxidase

Specific activity of peroxidase of *O. sativa* plantlets was determined after two weeks of culture on basal MS medium. As illustrated in Fig. 6, an overall increase in the specific activity of peroxidase was observed with increasing doses of ion beam. Non-irradiated plantlets were found to have specific activity of peroxidase of 647.67 U/mg, which was the lowest among all the treatments. On the other hand, the highest specific activity of peroxidase (1025.42 U/mg) was recorded in plantlets irradiated with 120 Gy of carbon ion beam, which was significantly greater than that of the control samples. Meanwhile, plantlets at 100 Gy also exhibited relatively high specific activity of peroxidase, which was 998.19 U/mg. This study also revealed that plantlets irradiated at 10, 20, 40, 60, and 80 Gy showed greater specific activity of peroxidase of 0.85%, 14.09%, 9.30%, 11.14%, and 30.45%, respectively, over the control samples. However, statistical analysis showed that the specific activity of peroxidase was not significantly different between all the treatments.

Besides being a source of stress to the plant, heavy ion radiation generates free radicals, which might be inhibitory to the biochemical processes (Verma *et al*., 2009). By knowing that water radiolysis as the predominant effect of radiation in organisms which induces reactive oxygen species (ROS) formation, one can assume that plant enzymes that are involved in cell protection against oxidative stress will display similar responses under radiation stress as under other stress factors (Zaka *et al*., 2002). According to the research conducted by Zhang *et al*. (2008), heavy ion obviously enhanced ROS, which is reflected by the increased production of O_2 and H_2O_2. Consequently, plants need to develop antioxidant

enzymes and antioxidative molecules to mitigate and repair the damage initiated by free radicals or ROS (Verma *et al*., 2009). In this case, peroxidases play an important protective role against oxidative stress and they are the indicators of cellular damage (Hameed *et al*., 2008).

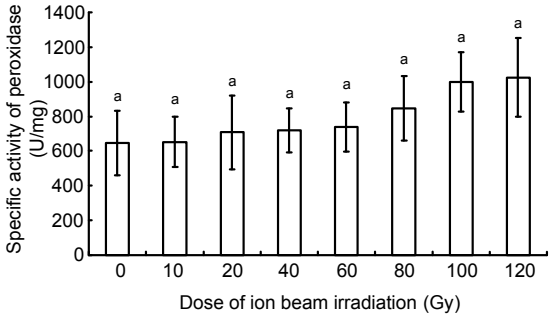

Fig. 6 Effects of carbon ion beam irradiation on the specific activity of peroxidase of *O. sativa* after two weeks of culture

Means with different letters are significantly different between treatments by the Tukey's HSD test ($P<0.05$). Error bars indicate the mean±standard deviation ($n=10$)

Peroxidase is one of the antioxidant enzymes that play an important role in plant defense systems (Veitch, 2004). In this study, it has been demonstrated that the specific activity of peroxidase increased with increasing doses of carbon ion beam. All plantlets derived from irradiated seeds were found to have higher specific activity of peroxidase as compared to the control samples. Current findings were supported by Verma *et al*. (2009) who revealed that there was a remarkable increase in peroxidase activity observed in the 30 and 90 d old *Brassica juncea*, with increasing doses of Li^{+} heavy ion administered. In a study to investigate the effects of ion irradiation on peroxidase abundance, activity, isozyme patterns, and transcription, Li *et al*. (2009) found an increased peroxidase activity, transcription, and translation as a result of seed irradiation of *Arabidopsis thaliana*.

Previous studies conducted on the effect of ionizing radiation on the peroxidase activity in a plant model also showed similar findings as observed in this study. Zaka *et al*. (2002) demonstrated enhanced activities of peroxidase, catalase, superoxide dismutase, and other antioxidative enzymes in *Stipa capillata* upon treatment with an external γ irradiation. Meanwhile, Cho *et al*. (2000) discovered that a group of genes, which includes glutathione-*S*-transferase, peroxidase, superoxide dismutase, and catalase, showed stimulation of transcript levels upon γ irradiation of young tobacco plants. This suggests that there was an increase in expression of peroxidase and its activity in young tobacco plants which were subjected to radiation stress. Enhancement of peroxidase activity in garlic (*Allium sativum* L.) was also reported as an effect of γ irradiation (Croci *et al*., 1991).

3.7 Chlorophyll content

Biochemical assay for the determination of chlorophyll content of the plantlets was conducted after two weeks of culture. In this study, it was demonstrated that the *O. sativa* plantlets derived from 10 Gy irradiated seeds had similar chlorophyll content as the control samples (Fig. 7). At 40 Gy of irradiation doses, the highest chlorophyll content among all treatments was recorded. A reduction in chlorophyll content was observed with the subsequent increase in irradiation doses. Currently, there is little research evidence available to support present observation of increased chlorophyll content as a result of irradiation. However, Creanga *et al*. (2005) found a stimulatory effect of small radiation doses on leaf chlorophyll content, upon the irradiation of the seeds of beans. Likewise, Singh (1971) found a significant increase in chlorophyll content of maize leaves following γ radiation exposures of 86.96–347.80 Gy.

In accordance with the present findings, Kalimullah *et al*. (2003) reported a decrease in chlorophyll content in rice seedlings irradiated by ^{1}H heavy ion, which may be due to the inhibition of chlorophyll biosynthesis by ^{1}H heavy ion beam irradiation. On the other hand, Bae *et al*. (2001) observed a drastic reduction in the chlorophyll and carotenoid contents of albino tobacco plants induced by ion beams. The chlorophyll and carotenoid contents of the mutant were found to be only 0.06% and 2.04%, respectively, of the levels in wild-type plants grown under similar conditions. It was also shown that exposure to ^{7}Li ions at 45 MeV resulted in an alteration in the energy level of various electron transport components within the photosynthetic apparatus (Gaikwad *et al*., 1999). Irradiation was found to affect chloroplast development, presumably via the disruption of plastid gene

expression (Bae *et al*., 2001), while photosynthetic pigments may be destroyed at high irradiation doses, leading to the loss of photosynthetic capacity (Strid *et al*., 1990).

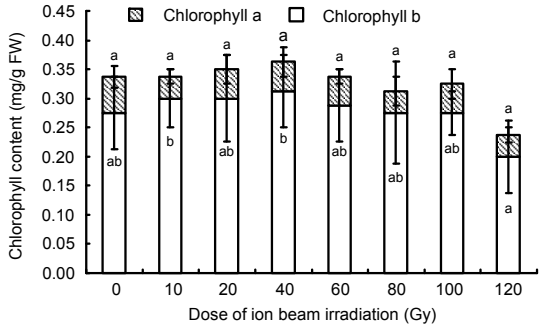

Fig. 7 Effects of carbon ion beam irradiation on the chlorophyll content of *O. sativa* after two weeks of culture
Means with different letters are significantly different between treatments by the Tukey's HSD test (P<0.05). Error bars indicate the mean±standard deviation (n=10)

In this study, it was also observed that all irradiated plantlets exhibited lower chlorophyll b content than the control samples. According to Creanga *et al*. (2005), the inhibition of chlorophyll b biosynthesis in irradiated plantlets was found to be more evident than that of chlorophyll a. This is probably due to a disturbance in chlorophyll b biosynthesis or degradation of its precursors as a result of irradiation (Saha *et al*., 2010). Current findings were also supported by Byun *et al*. (2002) who discovered the destruction of chlorophyll b by irradiation at 20 kGy.

Chlorophyll deficient mutants were frequently observed in studies involving ion beam irradiation of plant materials. Zhou *et al*. (2006) discovered a chlorophyll-deficient mutant which could transmit the character of chlorophyll deficiency to its progeny through three continuous culture cycles as a result of irradiating leaf explants of *Saintpaulia ionahta* with 5 Gy of 221 MeV carbon ion beam. Chlorophyll mutants were also evident in M_2 seedlings of carbon and helium ion irradiated tomato seeds (Masuda *et al*., 2009). Likewise, Abe *et al*. (2002) discovered chlorophyll deficient mutants with albino, pale-green, yellow or stripped leave phenotypes in M_2 progeny of rice as a result of carbon ion irradiation. However, Yamaguchi *et al*. (2009) reported that there were no differences in the spectrum of chlorophyll mutations between ion beams and γ rays, whereby, both high LET ion beam and low LET γ rays were found to induce albina, xantha, and other mutants such as striata (longitudinal white or yellow strips) and maculate (green or yellow spots distributed over the leaf) chlorophyll deficient mutants.

3.8 Total soluble nitrogen content

The total soluble nitrogen content of *O. sativa* plantlets was determined after two weeks of culture. As shown in Fig. 8, the total soluble nitrogen content of *O. sativa* plantlets varied with the doses of the ion beam administered. However, statistical analysis indicated that the total soluble nitrogen content was not significantly different between all the treatments.

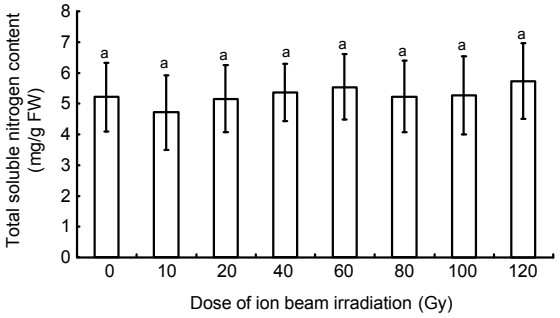

Fig. 8 Effects of carbon ion beam irradiation on the total soluble nitrogen content of *O. sativa* after two weeks of culture
Means with different letters are significantly different between treatments by the Tukey's HSD test (P<0.05). Error bars indicate the mean±standard deviation (n=10)

Nitrogen is required by plants in greater quantities than any other mineral elements to support their growth and development (Kirova *et al*., 2005). Based on the results of present investigation, higher total soluble nitrogen content was observed in plantlets derived from irradiated seeds as compared to the control samples. However, there was an exception for plantlets derived from 10 and 20 Gy irradiated seeds which exhibited relatively low total soluble nitrogen content as compared to the control samples. This was similar to the findings of Jones *et al*. (2004) who discovered a lower content of nitrogen in the shoots of *Holcus lanatus* that were exposed to 0, 5, and 10 Gy than in plants irradiated with 20 Gy and above. This may suggest a dilution effect resulting from uninhibited growth at the lower doses (Jones *et al*., 2004).

A literature search showed that there is no research evidence available regarding the effect of ion beam irradiation on the total soluble nitrogen content in plant systems. However, Chia (2008) observed higher total soluble nitrogen content in γ-irradiated *Citrus sinensis* plantlets, which was similar to the results obtained in the current study. On the other hand, Nassar *et al.* (2004) reported a clear effect of pre-sowing γ-irradiation on the nitrogen level of chamomile shoots and flowers. The highest nitrogen content was found in 60 Gy γ-irradiated chamomile shoots and 20 Gy γ-irradiated chamomile flowers. Similarly, Moussa (2006) reported an increase in nitrogen content, observed in 20 Gy of γ-irradiated rocket plants (*Eruca vesicaria* subsp. *sativa*). Meanwhile, Maltseva and Kuzin (1975) noticed an increase in nitrogen content when *Vicia faba* seeds were irradiated with 1 and 100 Gy of γ rays. Increased nitrogen and phosphorus contents as a result of irradiation were also observed in cabbage, onion, and carrot (Rennie and Nelson, 1975). According to Jones *et al*. (2004), high doses of irradiation sufficiently damaged the plants, leading to a reduced rate of nitrogen metabolism and mobilization relative to healthy tissues, thereby causing apparently greater and more constant nitrogen content. On the other hand, Bajaj (1970) suggested that the increase in total soluble nitrogen could be due to the quantitative changes in non-protein nitrogen or the accumulation of catabolic tissue products.

4 Conclusions

In short, the morphological and physiological responses of *O. sativa* towards carbon ion beam are dose-dependent. Low irradiation doses at 10 to 20 Gy stimulate growth and rooting ability of *O. sativa*, while irradiation at 20 to 40 Gy resulted in an increase in total soluble protein and peroxidase activity. Therefore, it is postulated that irradiation administered at low to moderate doses of 10 to 40 Gy may induce *O. sativa* mutants with superior characteristics. It is proposed that further investigations be carried out on the M_2 and M_3 progeny of *O. sativa* plantlets in order to identify the possible mutants induced by irradiation. This is because some of the mutated characteristics induced by carbon ion beam irradiation may not be evident in M_1 progeny of *O. sativa* plantlets. In addition, this will also help to determine whether the effect of carbon ion beam irradiation on *O. sativa* is transmittable to the succeeding generations.

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Compliance with ethics guidelines

Anna Pick Kiong LING, Ying Chian UNG, Sobri HUSSEIN, Abdul Rahim HARUN, Atsushi TANAKA, and Hase YOSHIHIRO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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