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Effects of Carbon Ion Beam Irradiation on Phenotypic Variations and Biochemical Parameters in Early Generations of Soybean Plants

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Abstract: Understanding the characteristics of carbon ion beam irradiation-induced mutation is essential to its potential application in plant breeding. A carbon ion beam-mutagenized soybean population was generated from the newly released soybean variety Dongsheng 28, with irradiation dosages of 100 Gy, 120 Gy and 140 Gy. Many phenotypic variations and novel mutants with heritable tendencies including plant height mutants, sterile mutants, early mature mutants, rolled leaves and short petioles mutants, yield-related mutants and lodging-resistant mutants were identified. Diverse variations in seed size, seed protein and oil concentration were found. Increasing irradiation dosage from 100 Gy to 140 Gy increased leaf chlorophyll concentration in M_1 generation, but this effect was significantly reduced in M_2 generation. The activities of superoxide dismutase (SOD), peroxidase (POD) and malondialdehyde (MDA) concentration all showed wider variation in M_1 and M_2 generation, the only exception being that the MDA concentration was similar to the control in the M_2 generation. Overall, we suggest that treating soybean seeds with carbon ion beam irradiation at a dosage of 120 Gy (80 Mev/u) could be effective in soybean mutation breeding.

Keywords: antioxidant enzymes; carbon ion beam; chlorophyll; mutant; phenotype; seeds composition; soybean

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1. Introduction

Genetic mutation provides the possibility of creating new genotypes in which breeders can identify appropriate phenotypes with the desired genes [1]. In nature, the natural mutation rate is about 0.1%, while the use of mutagenic agents such as radiation and chemicals can increase the mutation rate to up to 3%, 100 or even 1000 times higher than natural mutations [2]. Thus, mutation breeding has been widely applied to create crop varieties and desired mutants [3]. So far, according to the database from the Joint Division of the Food and Agriculture Organization of United Nations and the International Atomic Energy Agency (FAO/IAEA, Viena, Austrial), 3299 mutants have been released (http://mvd.iaea.org/).

In general, there are two types of mutagens, chemical mutagens and physical mutagens. Ethyl methane sulfonate (EMS) is one of the most intensively used chemical mutagens because the required method is easy to implement, does not require complicated equipment and has high frequency point mutations [4]. Espina et al. [5]. and Li et al. [6] constructed an EMS mutant population in soybean and found diverse phenotypes, including seed shape and composition and plant morphology and maturity. Moreover, there was evidence that these traits can be stably inherited. However, chemical mutagens are generally carcinogenic since low-efficiency and low-toxicity chemicals are not available. Therefore, physical mutagenesis is more often used in plant breeding [7]. The earliest radiation-induced mutation breeding was implemented by American scientist L. J. Stadler, who first confirmed the induced effect of x-rays on barley in 1928 [8]. Gamma rays, x-rays, neutrons and lasers are the most commonly used physical mutagens. In soybean alone, many types of mutations have been created through these mutagens, including mutants with high oil and protein concentration of seeds [9], high yield and early maturity mutants, seed color mutants and lipoxygenase-lacking mutants [10].

Carbon ion beam radiation (CIBR) is a new physical mutagen with high linear energy transfer (LET) and relative biological effectiveness (RBE) in biological systems [11], which has been increasingly applied to mutagenesis breeding in various plants. Compared to other mutagenic resources, the most prominent characteristic of CIBR is that it can release a large amount of energy at the end of the target material by forming a sharp energy shape, which demonstrates a higher mutation frequency and broader spectrum [12,13].

Changes in germination rate, emergence rate, plant morphology, fertility, quality and other traits have been found by CIBR in arabidopsis, tobacco, rice and other crop plants [14,15]. Changes in DNA base and ribose, strand breaks, cross-linking with proteins and a higher mutation rate were also reported [16–18]. Furthermore, many novel mutants, which are barely identifiable by other mutagens have been obtained by CIBR, such as pink, red and yellow petals in carnations, the number of petals, flower size, shape and color mutants in roses, complex and striped color mutants in chrysanthemums [19] and multifarious compound leaves mutants and chlorophyll deficiency mutants in soybean [20]. Though phenotype variation is the most common method for screening mutants, many traits are not visually observed and thus cannot easily be screened out. Therefore, it is necessary to properly combine the detection of biochemical parameters [21].

Similar to other stress factors, radiation breaks the dynamic balance of free radical and reactive oxygen species (ROS) production and elimination in plants. In order to avoid or reduce the harmful effects of ROS, plants develop enzymatic antioxidants like superoxide dismutase (SOD) and peroxidase (POD) [22–24]. There is also evidence that proline (Pro) accumulation can reduce ROS levels [25]. In addition, the change of malondialdehyde (MDA) concentration, the most important product of membrane lipid peroxidation, is a good and commonly used response indicator under stress conditions [26]. In general, lower doses of ionizing radiations played a modulatory role for the growth and developmental processes of seedlings while higher doses of exposure induce growth inhibition [27,28]. With an increase of the radiation dose, the activity of antioxidant enzymes and the concentrations of MDA, proline and hydrogen peroxide were found to increase, but when the radiation dose increased to a certain extent, these related indicators decreased or changed slightly [26,29,30].

In this study, the emergency and survival rates induced by CIBR for soybean variety Dongsheng 28 were investigated and the morphological and agronomic traits, seed morphology and seed composition in this mutant population were identified. In addition, biochemical parameters and their distribution in M_1 and M_2 plants in response to different irradiation doses were also examined. We anticipated that the determination of the optimal dose for mutagenesis and an understanding of early mutation effects could accelerate the application of CIBR in creating desired mutants and phenotypes in soybean breeding programs.

2. Materials and Methods

2.1. Plant Materials and Irradiation Treatments

The soybean variety Dongsheng 28 was used in this study. This variety has a high yield potential with wide adaptability and was released by the Northeast Institute of Geography and Agroecology of the Chinese Academy of Sciences. The irradiation treatments were conducted at the Institute of Modern Physics of the Chinese Academy of Sciences over two years (2017 and 2018). Dried soybean seeds of Dongsheng 28 with uniform size were chosen and divided into four groups of 100 seeds each to receive different doses of irradiation. The irradiation was set at 40 Gy per min with dosages of 0 (control), 70, 90 and 110 Gy in 2017 and 0 (control), 100, 120 and 140 Gy in 2018. The irradiation was directly targeted at the hilum of each seed with an 80 Mev/u carbon ion beam.

The treated seeds were then planted in a field at the Agronomy Farm of the Northeast Institute of Geography and Agroecology of the Chinese Academy of Sciences (45°73.2′ N, 126°61.2′ E) in May. Each treatment of 100 seeds was sown in a single row 5 m long, with a row spacing of 45 cm and plant spacing of 5 cm. The field was typical black soil with 29.3 g kg^{-1} organic matter content, 2.4 g kg^{-1} total N content, 1.5 g kg^{-1} total P content and 18.8 g kg^{-1} total K content. Before seeding, 70 kg ha^{-1} diammonium phosphate, 98 kg ha^{-1} urea and 120 kg ha^{-1} potassium sulfate base fertilizers were applied. The experiment followed local recommended management methods for weed control and other agronomic practices. All survival plants were harvested separately in the M_1 generation and every plant was sown in a single line in the M_2 generation.

In the M_1 generation, the number of seedlings was counted at 21 days after sowing and then the emergence rates were calculated. After emergence, all seedlings were observed every 2–3 days until the full maturity stage (R8); the survival rates were calculated by the number of surviving plants, including sterile plants, at different irradiation dosages. We measured plant height, branch number, node number, main stem diameter and bottom pod-setting height. The number of unproductive pods, number of productive pods per plant, number of seeds per plant, seed weight per plant, seed length and seed width per plant (the average of at least ten seeds randomly selected from each plant) were also measured. The seed protein and oil concentrations of all surviving plants that had at least 12 g of seeds were assayed by near infrared (NIR) spectroscopy (Perten IM9500, Sweden).

In the M_2 generation, the emergence rates and survival rates of different single lines were calculated. For each single line, five seeds were harvested from every individual plant to form a block for the determination of seed protein and oil concentrations. Only mutants with changes in traits were harvested as individual plants and the same parameters were measured as in the M_1 generation. These plants were continuously planted in the M_3 generation.

2.2. Measurement of Biochemical Parameters

Sampling. At the R2 stage (one open flower at one of the two uppermost nodes on the main stem with a fully developed leaf), leaves on the same node (the third node counted from top to bottom) from all surviving plants were sampled from 9:00 am to 10:00 am local time in the M_1 generation, while leaves of ten control plants were chosen randomly. Samples were put into a 10 mL centrifuge tube, frozen in liquid nitrogen and then stored in an ultra-low temperature freezer (−80 °C) for biochemical parameter determination. In the M_2 generation, five plants were randomly selected from each plant line and then the leaves on the third node were sampled and wrapped together.

Extraction. The extraction of leaf samples for biochemical parameters was conducted following the methods of Asghar et al. [29] and Dhindsa et al. [31]. Briefly, about 0.5 g of leaf sample was homogenized in phosphate buffer (pH = 7.8) and centrifuged at 10,000 rpm for 20 min at 4 °C. The supernatant was collected and used for the determination of SOD and POD activities and MDA concentration.

SOD Determination. Phosphate buffer (pH = 7.8), 130 mM methionine (Met), 750 μM nitro blue tetrazolium chloride (NBT), 100 μM EDTA-Na_2, 20 μM riboflavin (FD) and H_2O were mixed at a ratio of 15:3:3:3:3:2.5 for the extraction of SOD. Then, 20 μL supernatant was added to 3 mL mixed liquid in a 10 mL centrifuge tube and placed under light at 4000 lux for 30 min, before the reaction was terminated in the dark. The absorbance of the solution was read at 560 nm with a spectrophotometer (XinShiJi T6, Beijing, China). One unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of nitro blue tetrazolium reduction.

POD Determination. A total of 28 μL guaiacol was added to 50 mL phosphate buffer (pH = 6.0), dissolved by magnetic stirring, cooled and uniformly mixed with 19 μL of 30% H_2O_2. Then, 20 μL supernatant was added to 3 mL mixed liquid and changes in absorbance of the reaction solution at 470 nm were determined every 30 s.

MDA Concentration Determination. Homogenized 1 mL supernatant and 2 mL 5% TBA in a 10 mL centrifuge tube were placed in a boiling water bath for 15 min, centrifuged at 4000 r/min for 10 min and then the supernatant was subjected to absorbance at 600 nm, 532 nm and 450 nm.

Chlorophyll Concentration Determination. Chlorophyll was determined using a method similar to that of Einhellig [32]. About 0.3 g of fresh-weight leaf sample was put into a 50 mL centrifuge tube and 15 mL of 95% ethyl alcohol added in sequence, which was then extracted over 24 to 36 h in the dark. The extract was then diluted ten times with 95% ethyl alcohol and the absorbance of the extracts was measured at 665 nm, 649 nm and 470 nm by spectrophotometer.

2.3. Statistics Analysis

One-way ANOVA ($p < 0.05$) was performed to analyze the differences in emergence rates and survival rates between irradiation doses. A Box plot was created to compare the differences among other physiological indicators. The box plot plots the median, 10th, 25th, 75th and 90th percentiles as vertical boxes with error bars. Each box contains the data of a survival plant at every irradiation dose. The figures were all created with Graphpad Prism8.

3. Results

3.1. Effects of CIBR on Emergence Rate and Survival Rate in the M_1 and M_2 Generations

Our previous report suggested that 100 Gy of carbon ion beam irradiation could be applied in soybean mutation breeding through the screening of 100, 150, 200, 300 and 400 Gy dosages [33]. In 2017, we further refined the experiment with irradiation doses of 70, 90 and 110 Gy. Though there was no significant difference between the control (78%) and 110 Gy treatment (71%) for the emergency rate ($p < 0.05$), the values of the emergence counts minus the final plant counts for the irradiance treatments of 70, 90, and 110 Gy were 49%, 44% and 62%, respectively; higher than that of the control (5%). Compared to 2017, the emergence rate in 2018 decreased obviously. Significant differences were found between the control and 100 Gy treatments for both emergence rate and survival rate, while the decreases in the 120 Gy and 140 Gy treatments were not significant. Although no significant difference was found between 120 Gy (33%) and 140 Gy (31%) for the emergence rate, the survival rate of 140 Gy, at 18%, was obviously lower than that of 120 Gy, at 27% (Figure 1).

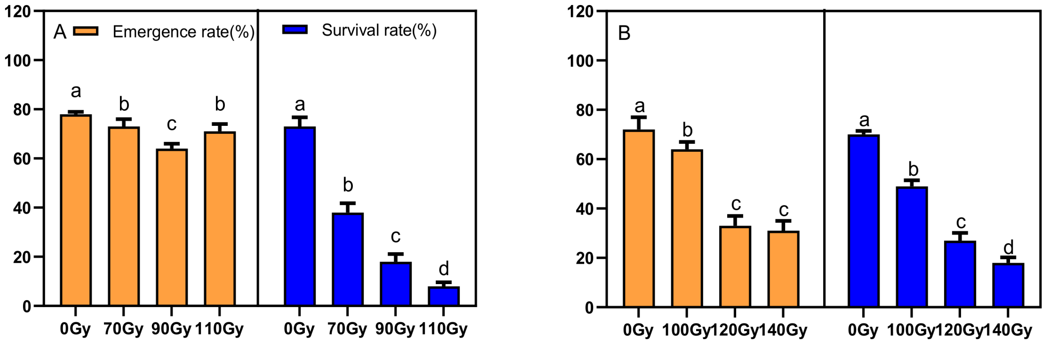

Figure 1. Effects of $^{12}C^{6+}$ carbon ion beam irradiation on emergence rate and survival rate in the M_1 generation. Means with different letters are significantly different at $p \leq 0.05$. (**A**) The emergence rate and survival rate of different irradiation doses in 2017. (**B**) The emergence rate and survival rate of different irradiation doses in 2018.

Irradiation-induced mutation also affected the emergence rate and survival rate in the M_2 generation. In order to better compare the effects of different radiation doses on the emergence rates from different family lines, we divided the emergence rate into five levels, as shown in Figure 2. In 2017, only 50% of the lines showed higher emergence rates of 80% to 100%, and in 2018, with the increase in irradiation dose, the percentage of the lines with an emergence rate over 80% decreased to 20%.

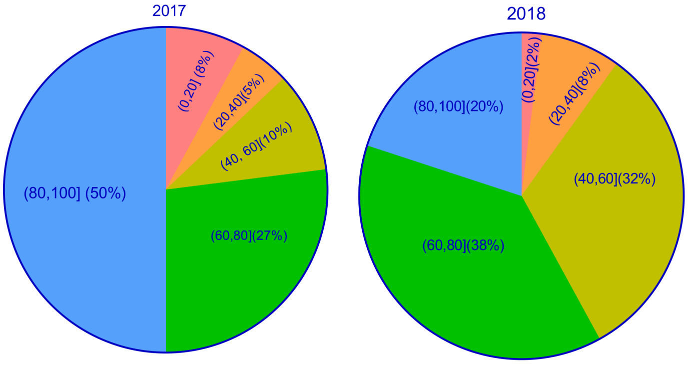

Figure 2. Emergence rate percentages in M_2 families.

3.2. *Screening for Visual Morphological Traits Mutants*

In the M_1 generation, compared to the control plants, the emergence of radiation-treated seeds was delayed by 3–5 days and the plants grew more slowly during the seedling stage. However, this phenomenon disappeared in the M_2 generation and many mutants with visible phenotype changes were identified. The most striking phenotypic variations observed during the growing period were altered leaves and stems, including pentafoliate leaves (Figure 3C,D), tetrafoliate leaves (Figure 3E), deformed leaves mutant (Figure 3F), the mutant with bent downward leaves (Figure 3G) and the mutant with double main stem (Figure 3I). With regard to mutants with multicompound leaves, the leaves over the whole plants were all pentafoliate leaves or tetrafoliate leaves. Unfortunately, these mutants were all dead before the pod-setting stage. Many plants showed the traits of double main stems (Figure 3I) in the M_1 generation, but these traits disappeared in the next generation. In the M_2 generation, at the seedling stage, we found many seedlings with two pairs of unifoliolate leaves (Figure 3A) and yellowing of unifoliolate leaves (Figure 3B). Thickening and shrinkage of cotyledons were also observed but they could have been useless variations and we did not propagate them in the next generation.

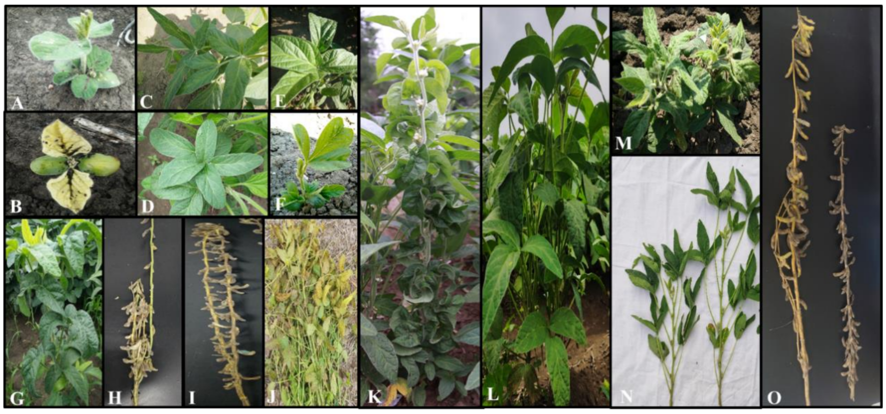

Figure 3. Examples of phenotypes observed in $^{12}C^{6+}$ carbon ion beam-induced soybean population. (**A**) Yellowing unifoliolate leaves mutant. (**B**) Two pairs of unifoliolate leaves mutant. (**C**,**D**) Pentafoliate leaves mutant. (**E**) Terafoliate leaves mutant. (**F**) Deformed leaves mutant. (**G**) Leaves bent downward mutant. (**H**) Multibranches mutant. (**I**) Double main stem mutant. (**J**) Sterile plants mutant. (**K**) Rolled leaves and short petioles mutant. (**L**) Control plants. (**M**) Multileaves mutant. (**N**) Short height mutant (on the right is the control). (**O**) Short growth period mutant (on the left is the control).

We also observed many variations that had genetic predispositions. As shown in Figure 3K, we found a mutant with the leaf curled downwards at 90 Gy in the M_1 generation. This mutant had a very short petiole, erected at the lower part and crept at the upper part of the plant, which still showed character separation until the fourth generation. Furthermore, in one line we found mutants with narrow and slender leaves but plant heights less than

20 cm (Figure 3M). On the same plants, trifoliate, pentafoliate and hexafoliate existed simultaneously and the plants could flower normally but could not bear seeds. We also found sterile plants in two lines (Figure 3J) and a large number of sterile plants were still found in the third generation. Surprisingly, we found a special mutant population in which there was significant separation in growth period traits and plant height traits in the M_3 generation: the plant height was reduced by about 50% and the growth period was either 15 days longer or 15 days shorter than normal (Figure 3N,O). We also screened out eight yield-related and 15 lodging-resistant candidate mutant lines, since environmental factors have a greater impact on these traits and genetic stability would need to be verified.

3.3. Effects of Carbon Ion Beam Radiation on Seeds Size and Seed Composition

In order to better quantify the changes in seed morphology and composition of the mutagenized progeny, we measured the seed length, seed width and the concentrations of seed protein and oil for the individual plants in 2018. The M_1 population generally showed significant variations in seed traits (Figure 4). Generally, the irradiation treatments produced larger seeds, with seed lengths ranging from 7.5 mm to 8.0 mm, and seed width from 6.0 mm to 7.5 mm, compared to the seeds of the control plants (7.20 mm to 7.52 mm seed lengths and 6.19 mm to 6.58 mm seed widths). The 120 Gy treatment resulted in the most extensive variation (Figure 4A,B). The radiation treatments increased the number of unproductive pods, one-seed pods and two-seed pods, but decreased the number of three-seed pods and four-seed pods, compared to the control. As shown in Figure 4C, the ratio of seed to pod was greater (2.5 to 3.5) for the control than for the irradiation treatments (1.0 to 2.5). Surprisingly, the increase in the number of one- pod and two-seed pods did not lead to a decline in yield due to higher number of pods with heavier seeds in the mutants. For example, one mutant produced 94 one-seed pods and 42 two-seed pods and its single plant seed weight was 52.79 g, which was 109% higher than the control plants. Another mutant resulting from the 140 Gy treatment had 61 one- and two-seed pods and 87 three- and four-seed pods, with the single plant seed weight reaching 77.53 g, which was about four times greater than the control. Many mutants with significantly altered seed sizes were also identified in the M_2 generation, including some of the mutants mentioned above. These mutants continued to show a tendency of stable inheritance in the M_3 generation. Thus, when the traits were heritable and stable in the later generation, they produced extraordinarily high yields and had larger seed germplasms.

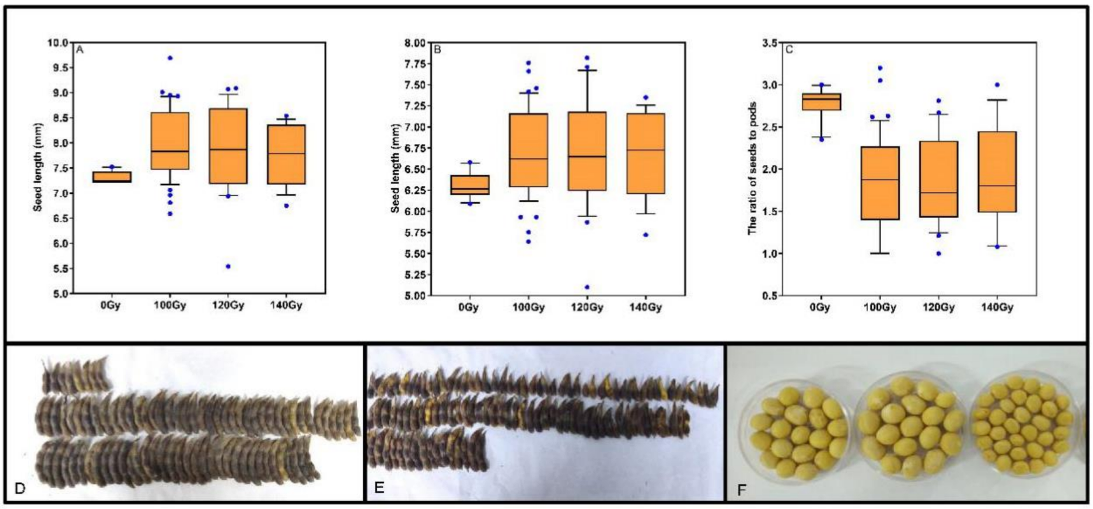

Figure 4. Distributions of seed length, seed width and ratio of seeds to pods in the M1 population. (**A**) Seed width; (**B**) seed length; (**C**) ratio of seeds to pods; (**D**) pods of WT; (**E**) pods of mutants with more one- and two-seed pods; (**F**) mutants with altered seed sizes (from left to right: WT, lager seeds mutant, smaller seeds).

For mutants that produced at least 12 g of seeds, we performed a, NIR analysis (Figure 5) to determine seed quality traits in the M_1 and M_2 generations. In the M_1 generation, the range of protein concentration was 35.1–46.5% and most of the plants had higher seed protein concentrations, especially for the higher irradiation dose of 140 Gy. In the M_2 generation, the range of protein concentration was 38.5–42.9% and the median values of the three irradiation doses were similar. In the M_1 generation, the oil concentration for the irradiation treatments also showed significant variation, ranging from 16.2–20.8%, but was lower in most of the plants. In the M_2 generation, lower oil concentrations at 100 Gy but concentrations at 120 Gy and 140 Gy, with a range of 18.8–23.8%, were found in most of the plants. The change of total protein and oil concentration in the M_1 generation was not obvious but in the M_2 generation higher total protein and oil concentration was found for higher irradiation doses (120 Gy and 140 Gy).

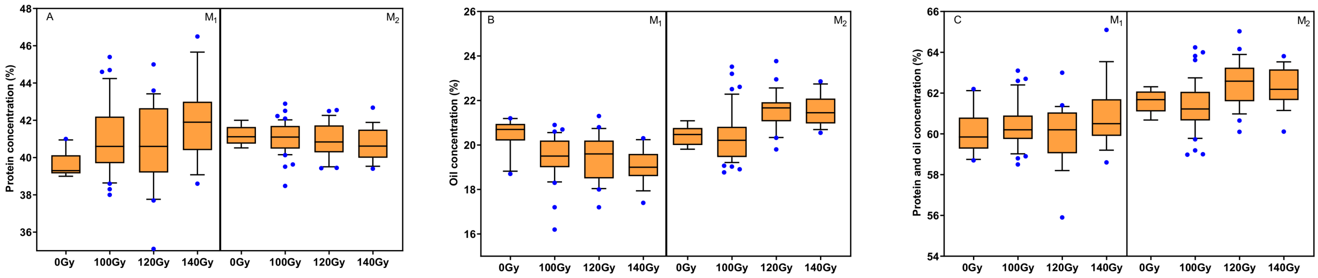

Figure 5. The distribution of seed protein concentration and oil concentration in the M_1 and M_2 populations. (**A**) Protein concentration; (**B**) oil concentration; (**C**) protein and oil concentration.

3.4. Effects of Carbon Ion Beam Radiation on Chlorophyll Concentration

The concentrations of chlorophyll-a, chlorophyll-b and carotenoid and the total chlorophyll in the M_1 and M_2 generations according to different dosages at the R2 stage are shown in Figure 6. In the M_1 generation, with the increase in irradiation dose, the chlorophyll-a concentration showed a decreasing tendency and all irradiation doses obviously increased the chlorophyll-b concentration and carotenoid concentration. For the total chlorophyll concentration, the median values were all higher for the irradiation treatments than for the control, though those of the 140 Gy treatment was lower than that of the 100 and 120 Gy treatments. In the M_2 generation, the ranges of chlorophyll-a, chlorophyll-b, carotenoid and total chlorophyll concentrations were not wide compared to the M_1 generation and the median values of chlorophyll-a and chlorophyll-b concentrations of the irradiation treatments were similar to the control. The median values of carotenoid concentration in the irradiation treatments were higher than that of the control, especially at 120 Gy. However, the concentrations of total chlorophyll in all irradiation treatments were lower than in the control, especially at 140 Gy.

3.5. Effects of Carbon Ion Beam Radiation on Antioxidant Enzyme Activities and MDA Concentration

In the M_1 generation, it was observed that 120 and 140 Gy irradiation treatments enhanced the activity of SOD, with 120 Gy showing a greater variability. In the M_2 generation, a wider range of SOD activities relative to the M_1 generation was found but the median values were all below the control (Figure 7A). Greater variability for POD activities in the M_1 generation was also found for the 120 Gy treatment and a larger proportion of POD activities were greater than the median for the 120 and 140 Gy groups. In the M_2 generation, the POD activities of irradiation treatment were generally higher than in the control (Figure 7B). The 120 Gy treatment in the M_1 generation induced greater variability for MDA concentrations and those over the median accounted for a larger proportion, but no similar variations were found at 100 and 140 Gy. In the M_2 generation, the variation of MDA concentrations in irradiation treatments was not wider than that in the M_1 generation (Figure 7C).

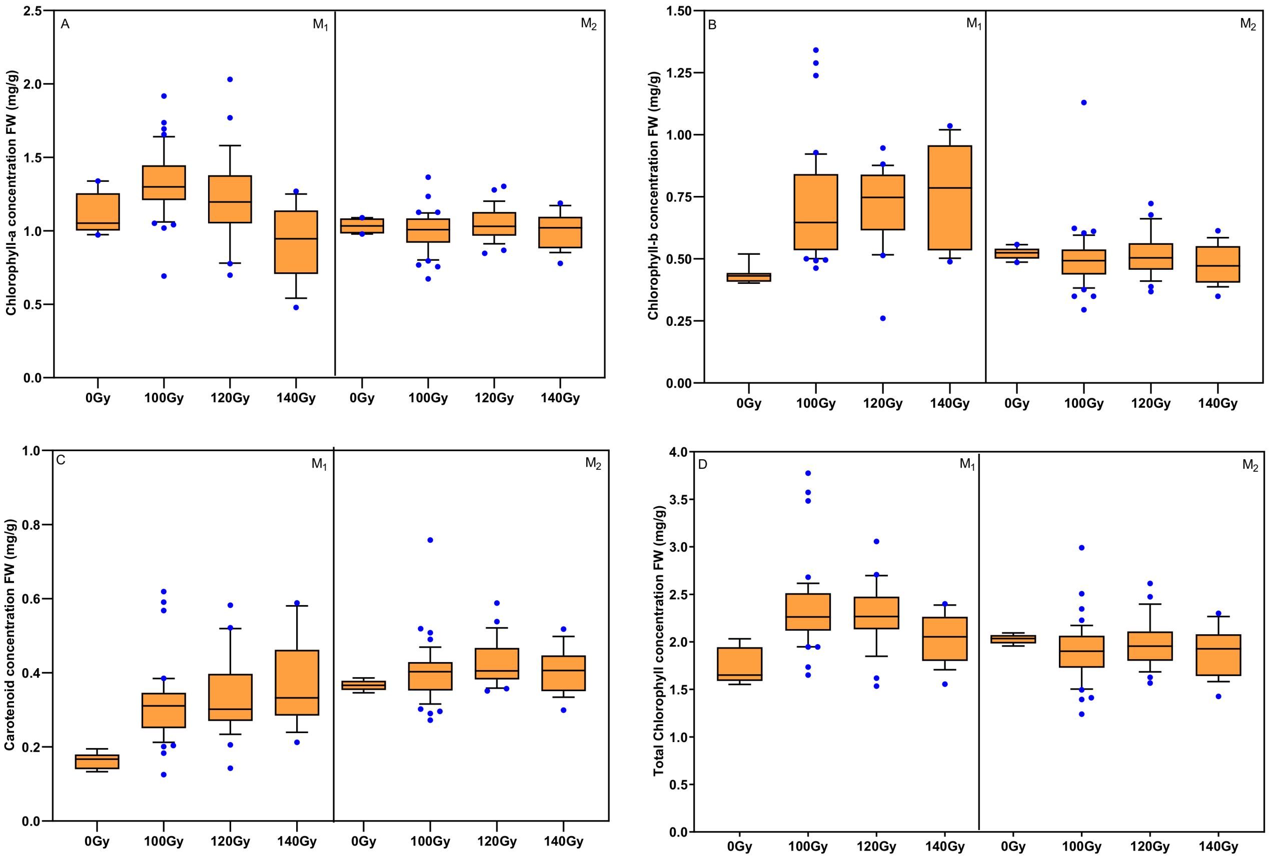

Figure 6. Chlorophyll-a, chlorophyll-b, carotenoid and total chlorophyll concentrations in the M_1 and M_2 generations at different irradiation dosages. (**A**) Chlorophyll-a. (**B**) Chlorophyll-b. (**C**) carotenoid. (**D**) Total chlorophyll

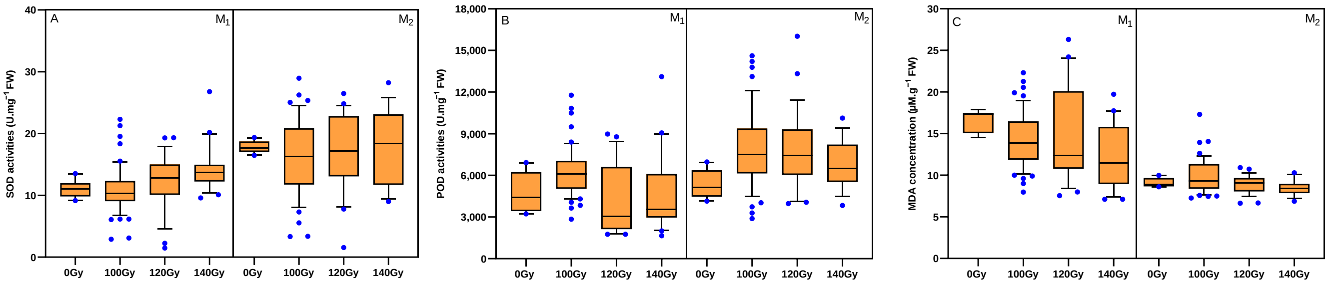

Figure 7. Effects of different radiation doses on antioxidant enzyme activities and MDA concentrations in the M_1 and M_2 generations. (**A**) SOD activities; (**B**) POD activities; (**C**) MDA concentration.

4. Discussion

The optimal radiation dose determines the success of mutation breeding [34]. In order to ensure high mutation frequency with more beneficial mutants and obtain sufficient viable seeds at the same time, a dose causing 50% lethality is often chosen as the optimal radiation dose [1]. Emergence and survival rates decreasing with an increase of the irradiation dose has been reported [35,36] but, in the present study, we found that the 70, 90 and 110 Gy treatments generally showed lower survival but higher emergence rates. We demonstrated that the range of 70 to 90 Gy might not be enough for getting sufficient mutants and that the lower survival rate is probably due to the weaker resistance of the seedlings after radiation and the combined influence of environmental factors. This was also supported by the significant decline of the emergence rate in the M_1 and M_2 generations from 120 to 140 Gy. We found that the 120 Gy treatment had a similar emergence rate to the 140 Gy treatment but a higher survival rate, which is more suitable for mutation breeding of soybean. Therefore, to obtain more mutants, higher doses might be a good choice for carbon ion beam irradiation.

The present study identified many novel phenotype mutants with heritable tendencies, including plant height mutants, sterile mutants, early mature mutants, rolled leaves and short petioles mutants, seed size alteration mutants, yield-related mutants and lodging-resistant mutants. Similar phenotype changes have also been also found with the EMS mutagen [5,6]. However, the mutant we found with rolled leaves and short petioles has never been reported. The mutants obtained in this study, not only provide germplasms resource for breeding, but also could be good materials for studying hormone metabolism, photosynthesis and growth habits, while the early maturity and lodging resistance candidate mutants are of great significance for the improvement of the original varieties.

Significant increases or decreases in seed protein and oil concentrations in mutants across multiple generations and environments as a result of radiation have been reported [9]. Higher concentrations of EMS were effective in increasing the variability for fatty acid content in soybean oil [37]. We also found that higher irradiation doses were more effective in increasing the oil concentration, as well as the total protein and oil concentration. The wider range of protein and oil concentrations in the M_1 generation was probably not inheritable; therefore, in soybean breeding, the selection of high-oil and high-protein mutants should start in the M_2 generation and CIBR could be a good approach to screening for germplasm resources with large seed sizes and high protein and oil concentrations.

Chlorophyll-a, chlorophyll-b and carotenoids are important pigments in photosynthesis [38] and they are also important biochemical indicators for measuring the degree of radiation damage in mutation breeding. Many investigations have indicated that radiation mutagenesis has a higher probability of screening for chlorophyll-related mutants [39,40]. In contrast to the results of Alilamamogul et al. [22], who found that chlorophyll-a, chlorophyll-b and total chlorophyll were negatively affected by radiation, our results showed that, except for a decreased concentration of chlorophyll-a in the 140 Gy treatment in the M_1 generation, the irradiation treatments consistently increased chlorophyll-a, chlorophyll-b, carotenoid and total chlorophyll concentrations. However, this effect was significantly decreased in the M_2 generation, which implies that the positive effect of irradiation on chlorophyll concentration in the M_1 generation may not be inherited in the next generation.

Physiological level variations caused by radiation reported by previous researchers were mostly concentrated in seeds or seedlings and demonstrated that ionizing radiation increased the activities of catalase (CAT), SOD and POD, which are well correlated with the enhancement of growth [29,41,42]. In our study, SOD and POD activities in the M_1 and M_2 generations both showed wider variation, especially at 120 Gy, which indicates that CIBR can, to a certain extent, improve the ability of some plants to protect against free radical damage. As a good and commonly used response indicator under stress conditions, MDA concentration is the most important product of membrane lipid peroxidation [26]. The MDA concentrations for irradiation treatments in our study showed diverse variability in the M_1 generation, but not in the M_2 generation, which indicated that the physiological damage caused by CIBR was significantly restored in the M_2 generation. In summary, we propose that the emergence rate, the survival rate, the beneficial mutation rate, environment conditions and some biochemical parameters should be all considered in order to determine the optimal dose of CIBR accurately. We will continue to observe the physiological changes in later generations to judge whether the determination of the biochemical indicator of the mutant population could be used as one of the criteria for screening the optimal radiation dose and the specific mutant.

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