Cytogenetic Effects of Low Doses of Energetic Carbon Ions on Rice After Exposures of Dry Seeds, Wet Seeds and Seedlings

Jin-Ming SHI1, Jian-Guo GUO1, Wen-Jian LI2, Meng ZHANG1, Lei HUANG3 and Ye-Qing SUN1,3*

Rice/Heavy ion radiation/Low dose/Chromosome aberrations/Embryo.

In order to investigate the biological effects of heavy ion radiation at low doses and the different radiosensitivities of growing and non-growing plants, rice at different lift stages (dry seed, wet seed and seedling) were exposed to carbon ions at doses of 0.02, 0.2, 2 and 20 Gy. Radiobiological effects on survival, root growth and mitotic activity, as well as the induction of chromosome aberrations in root meristem, were observed. The results show that radiation exposure induces a stimulatory response at lower dose and an inhibitory response at higher dose on the mitotic activity of wet seeds and seedlings. Cytogenetic damages are induced in both seeds and seedlings by carbon ion radiation at doses as low as 0.02 Gy. Compared with seedlings, seeds are more resistant to the lethal damage and the growth rate damage by high doses of carbon ions, but are more sensitive to cytogenetic damage by low doses of irradiation. Different types of radiation induced chromosome aberrations are observed between seeds and seedlings. Based on these results, the relationships between low dose heavy ion-induced biological effects and the biological materials are discussed.

INTRODUCTION

Biological effects of ionizing radiation (IR) have been studied for nearly a century due to the wide use of IR in medical diagnosis, cancer therapy and mutation research. Because of the power of IR as mutagen in producing novel mutants in agricultural applications and scientific researches, high doses of IR are often used and extensively investigated on their mutagenic effects. However, low dose ions are the major sources of radiation commonly encountered in the events of nuclear pollutions, medical treatments and in space flights. In investigations of biological effects of IR, plants can be excellent models since they are widely used for studying the effects of environmental stresses. With the development of space research, plants also become one of the important model organisms in assessing damages of the space radiation environment. Hence, understanding the response of plants to low dose and high-LET IR is important in space life sciences.

In plants, IR-induced biological effects were found in many endpoints including phenotypic mutation, cytogenetic aberration and DNA breaks. These damages can also be detected at very low dose. IR with a dose range of 10–100 mGy was proved to be effective in causing effects on plant growth. Previous studies in wheat revealed a remarkably strong induction of germline mutation after exposure to IR with a low dose of 0.3 Gy from the Chernobyl accident. Low dose of X-rays (1.25 Gy) also enhanced the relative recombination frequency to approximately twice the spontaneous value in Nicotiana tabacum somatic cells. Gamma irradiations of roots of Vicia faba induced micronuclei in root cells, which were observed at a dose range of 0–2.5 Gy. Disturbance of mitotic index (MI) of barley roots were observed after irradiated by gamma rays at doses below 1 Gy. Researches using gamma rays also found that IR at doses below 1 Gy is efficient in producing chromosome aberrations in seedling of the plants than expected. In the life cycle of higher plants, seed is of particular interesting. Using plant seeds as material, it has been proved that high-LET radiation effectively causes morphological and cytogenetic effects on plant seeds even at doses as low as 2 mGy.

The risk from exposures to IR is not only dependent on the dose of the radiation but also on the radiosensitivity of living organisms. Radiosensitivities are different for different cell types and tissues. For example, it is well known that...
cancer cells are more sensitive to IR than normal cells.\textsuperscript{23,24) The high radiosensitivity of embryo has also been proved, as evidence suggests that early embryos of mouse are much more sensitive to radiation than adults.\textsuperscript{25–27) In plants, radiation acting on embryos can induce more phenotypic mutations than other tissues in Arabidopsis seeds.\textsuperscript{3,9} Seeds and seedlings provide unique models to analyze different radiosensitivities between embryo and growing tissues in mitotic cycles. Comparative studies of the varying radiosensitivities of plants and their seeds often show that seeds are more resistant to IR induced biological effects.\textsuperscript{7,29} It has also been reported that differential effects on growth and phenotype were found between seeds and seedlings by gamma rays.\textsuperscript{29} However, most of the reported investigations use low-LET and high dose IR. Compared with low-LET, heavy ions of high-LET often possess greater potential to induce mutagenic effects even at lower doses.\textsuperscript{2,30,31} The main purpose of this paper is to reveal the responses of plants to low dose heavy ion radiation and to compare the radiosensitivity of plants at different life stages.

In studies concerning the damage effects of mutagenic agents, detections of cytogenetic effects such as micronucleus formation and chromosome aberrations were widely used since the 1930s.\textsuperscript{32} Chromosome aberrations detected in root meristem cells were recognized as an efficient short term genetic bioassay. In the present study, we measure the damages in chromosomes as well as the changes on the mitotic activity of the root tips to evaluate the IR induced biological effects on plants.

**MATERIALS AND METHODS**

**Plant material preparation**

Rice (Oryza sativa, japonica) seeds were obtained from Harbin Institute of Technology in Harbin, China. Wet seeds were obtained by dry seeds soaked in water at 25°C for 12 h. Seedlings were obtained by dry seeds soaked in water for 48 h at 25°C and then placed on moistened filter paper for 24 h at 25°C. The average root length when exposed to carbon ions is 0.5 cm.

![Radiation exposure of dry seed (a), wet seed (b) and seedling (c).](image)

Fig. 1. Radiation exposure of dry seed (a), wet seed (b) and seedling (c). After irradiation, rice was cultured for 108-hours until observation. Survival rate was evaluated at two weeks after dry seeds were soaked in water.

**Radiation treatment and ion parameters**

Irradiation experiment was performed using the equipment of Heavy Ion Research Facility in Lanzhou (HIRFL).\textsuperscript{33} Dry seeds, wet seeds and seedlings were horizontally positioned in a polystyrene chamber with a diameter of 3.5 cm during irradiation. The heavy-ion type used was 12C with the energy of 100 MeV/u. The mean LET within the rice was calculated to be 27.3 KeV/μm. The range of the incident ions in water was calculated to be 2.45 cm in water. In order to minimize the changes in LET, the path penetrated by the ions in dry seeds, wet seeds and seedlings were kept to be less than 0.9 cm, which is well in the plateau region of the carbon ion Bragg curve. Three kinds of rice materials were irradiated in the same condition with the doses of 0 Gy (control), 0.02 Gy, 0.2 Gy, 2 Gy and 20 Gy. The dose was measured by an air ionization chamber. The controls (0 Gy)
were handled in the same way. The experiment was repeated in the same condition.

Treatment after irradiation

Wet seeds and seedlings were washed with distilled water immediately. After irradiation treatment, seedlings were cultured on moistened filter paper at 27°C under 16 h light and 8 h darkness condition. Dry and wet seeds were soaked in distilled water at 25°C for 48 h and 36 h respectively and then treated as the seedlings.

![Fig. 3. Micronuclei and non-mitotic chromosome condensation (NMCC) on the root apical meristematic cells of rice induced by carbon ion exposure: (a) normal interphase; (b) normal prophase; (c) cell with NMCC in interphase; (d) cell with micronucleus. Arrow indicates a micronucleus. Bars = 10 μm.](image)

![Fig. 4. Carbon ion-induced mitotic chromosome aberrations (MCAs): (a) bridges in metaphase; (b) bridges in telophase; (c) fragments; (d) laggards; (e) stickiness; (f) uneven segregation and (g, h) disturbed spindle on root meristematic cells of rice. Bars = 10 μm.](image)

### Table 1. Frequency of micronuclei and non-mitotic chromosome condensations (NMCC) in the root meristem of rice induced by carbon ion irradiation.

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>Number of cells</th>
<th>Cells with micronuclei</th>
<th>Cells with condensed chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number per 1000 cells (mean ± SEM)</td>
<td>Number per 1000 cells (mean ± SEM)</td>
<td></td>
</tr>
<tr>
<td>Dry seed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10000</td>
<td>2</td>
<td>0.20 ± 0.13</td>
</tr>
<tr>
<td>0.02</td>
<td>8000</td>
<td>7</td>
<td>0.88 ± 0.22*</td>
</tr>
<tr>
<td>0.2</td>
<td>9000</td>
<td>11</td>
<td>1.22 ± 0.33*</td>
</tr>
<tr>
<td>2</td>
<td>8000</td>
<td>11</td>
<td>1.38 ± 0.26**</td>
</tr>
<tr>
<td>20</td>
<td>9000</td>
<td>22</td>
<td>2.44 ± 0.40***</td>
</tr>
<tr>
<td>Wet seed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10000</td>
<td>2</td>
<td>0.20 ± 0.13</td>
</tr>
<tr>
<td>0.02</td>
<td>9000</td>
<td>12</td>
<td>1.33 ± 0.36*</td>
</tr>
<tr>
<td>0.2</td>
<td>9000</td>
<td>15</td>
<td>1.67 ± 0.50*</td>
</tr>
<tr>
<td>2</td>
<td>10000</td>
<td>22</td>
<td>2.20 ± 0.49***</td>
</tr>
<tr>
<td>20</td>
<td>8000</td>
<td>35</td>
<td>4.38 ± 0.68***</td>
</tr>
<tr>
<td>Seedling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10000</td>
<td>3</td>
<td>0.30 ± 0.21</td>
</tr>
<tr>
<td>0.02</td>
<td>8000</td>
<td>3</td>
<td>0.38 ± 0.18</td>
</tr>
<tr>
<td>0.2</td>
<td>9000</td>
<td>6</td>
<td>0.67 ± 0.24</td>
</tr>
<tr>
<td>2</td>
<td>9000</td>
<td>28</td>
<td>3.11 ± 0.46***</td>
</tr>
<tr>
<td>20</td>
<td>9000</td>
<td>103</td>
<td>11.44 ± 0.79***</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01; ***P < 0.001.
dose, 30 were randomly chosen for root tip observation. The rest were kept in culture for the survival rate examination at 2 weeks after germination. For cytogenetic endpoints, rice grown from the exposed dry seed, wet seed, and seedling states were detected at the same growing stage. The root tips were collected at four and half days (108 h) after soaking in water (Fig. 1) and the root length was measured at that time. The root tips were fixed in the Carnoy's fixative solution (ethanol:acetic = 3:1), incubated in 1 M HCl and then stained with carbolic acid fuchsin. Identification of cytogenetic aberrations was performed according to the criteria of Wei et al., Fusconi et al., and Tkalec et al. A total of 30 root tips and 500–600 cells per root tip for each dose treatment were analyzed for mitotic index (MI). In the MI analysis, the mitotic cells were counted and the MI was calculated, and expressed as percentage, as the ratio between the number of mitotic cells and the total number of cells scored. A total of 8000–10000 interphase cells and 400 mitotic cells from at least six root tips of each replicate were analyzed for evaluation of micronuclei and mitotic chromosome aberrations (MCAs), respectively. For MCAs observation, the classification of aberrations was according to the aberration type observed in the root tip cells. The nomenclature of each aberration type was also consistent with other studies. The frequency of mitotic and MCAs was expressed as per-millage and percentage in relation to the observed cells.

**RESULTS**

**Effects of carbon ion irradiation on survival rate, root growth and MI**

Survival rates of rice after exposure at different doses were showed in Fig. 2A. At 20 Gy, a loss of viability in rice after exposures of wet seeds and seedlings was observed, but no significant lethal effect was observed in for the dry seeds after the 20 Gy exposure.

The root length and MI of the root tip cells after irradiations of dry seeds, wet seeds, and seedlings were showed in Fig. 2B and 2C. For the dry seeds, a slight decrease of the root length was observed, but no significant changes of MI were noticed. For the wet seeds and seedlings, exposures to carbon ions caused significant decreases of both the root length and MI were found at the highest dose. At 2 Gy, significant increases of the root length and MI were observed in the seedling assay (P < 0.01).

**Table 2. Frequency of mitotic chromosome aberrations in root meristems of rice induced by carbon ion radiation.**

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>Number of cells</th>
<th>Mitotic chromosome aberrations</th>
<th>Sun per 100 cells (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bridge</td>
<td>Fragment</td>
</tr>
<tr>
<td>Dry seed</td>
<td>0</td>
<td>400</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>400</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>400</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>400</td>
<td>6</td>
</tr>
<tr>
<td>Wet seed</td>
<td>0</td>
<td>400</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>400</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>400</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>400</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>400</td>
<td>9</td>
</tr>
<tr>
<td>Seedling</td>
<td>0</td>
<td>400</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>400</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>400</td>
<td>33</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.01; ***P < 0.001.*

Data analysis

Statistical analysis was performed using the Microsoft Excel. Differences between the means were determined using the t-test.
Cytogenetic Effects of Heavy Ion Radiation on Rice

Effects of carbon ion radiation on interphase aberrations and mitotic chromosome aberrations (MCAs)

Chromosome observations were performed in interphase cells of the root tip meristem. Besides micronuclei, radiation induced non-mitotic chromosome condensation (NMCC) were observed in both the seed and the seedling assays (Fig. 3).

As shown in Table 1, the percentages of cells containing interphase aberrations including micronuclei and NMCC increased with the increasing dose. Exposures at lower doses (0.02 and 0.2 Gy) can induce significant aberrations in both dry seeds and wet seeds. For the seedling assay, significant damages were only observed after exposures at higher doses (2 and 20 Gy).

In mitotic cells, many types of chromosomal damages were induced in both dry seeds, wet seeds and seedlings after radiation treatment (Fig. 4). As shown in Table 2, the frequency of mitotic chromosome aberrations was increased with the radiation dose. Significant changes in respect to the control (0 Gy) were also observed at low doses.

The differences of chromosome aberrations between exposures of dry seeds, wet seeds and seedlings at each dose were showed in Fig. 5. At lower doses, the percentages of cells with chromosomal damages in seeds (dry or wet) were higher than that in seedlings, while more chromosome aberrations were observed in seedlings than in seeds after higher dose exposures.

Carbon ion induced MCAs were divided into six types according to their characters including: 1) bridges, 2) fragments, 3) laggards, 4) stickiness 5) disturbed spindle, and 6) uneven segregations (as shown in Fig. 4). The percentages of each type of MCAs induced in the dry seed, wet seed and seedling assays were compared in Fig. 6.

DISCUSSION

Results in the present study suggest that biological responses to low dose IR are different from that to high dose IR. Exposures to low dose carbon ions induced stimulatory effects on root growth and mitotic activity of rice while exposures to high dose carbon ions displayed inhibitory effects. As showed in Fig. 2 (B and C), the root growth and MI of wet seeds and seedlings increased at after lower dose exposures, but deceased significantly with increasing doses. This phenomenon can be explained by radiation hormesis which is widely demonstrated by previous researches in plants as well as in other organisms including mammals, bacteria and fungi. Our results also showed positive effects...

Fig. 5. Effects of four radiation dose (0.02, 0.2, 2 and 20 Gy) on the root tips of rice after exposure at the dry seed, wet seed and seedling stages. Chromosome aberrations include micronuclei (MN), non-mitotic chromosome condensation (NMCC) and mitotic chromosome aberrations (MCAs). The columns present the frequency of chromosome damages higher than the control (0 Gy). The MN and NMCC were expressed as permillage of total number of interphase cells observed. The MCAs was expressed as percentage of total number of mitotic cells. Bars indicate the standard error of means.

Fig. 6. Comparison of mitotic chromosome aberrations (MCAs) induced by carbon ions. Each type of MCA was expressed as the percentage of all MCAs.
of lower dose and negative effects of higher dose of carbon ions on the root growth and the mitotic activity.

Comparison of radiosensitivities in the present study showed that both dry and wet seeds were more resistant than seedlings to radiation-induced lethal effect and mitotic activity changes. Between the dry and the wet seeds, wet seeds were more sensitive to these effects than dry seeds (Fig. 2). One of the differences between dry and wet seeds which may be responsible for the different radio-responses is the water content. Previous studies have shown that the water content at the time of irradiation play an important role in the radiosensitivity of seeds. For instance, Gustafsson et al. reported that seeds with higher water content were more susceptible to X-rays than seeds with lower water content.\(^{37}\) Qin et al. found that water-imbibed seeds of Arabidopsis are more sensitive to protons than dry seeds.\(^{39}\) Biebl et al. also demonstrated an optimum water content at which the radiosensitivity of seeds reached its minimum.\(^{38}\) In the present study, the dry seeds and water-imbibed seeds (wet seed) of rice were exposed to carbon ion beams. The results proved that at this condition, the wet seeds of rice were more sensitive to carbon ion-induced damages than the dry seeds.

In Fig. 5, it was shown that more chromosome damages were observed in seeds (dry and wet) than seedlings especially at doses of 0.2 Gy and lower. These results suggest that seeds are more sensitive to carbon ion-induced chromosomal damages than seedlings at lower doses. This phenomenon may be induced by the action of energetic particles on the embryo inside the seeds. A seed is a special stage of plant’s life cycle. On one hand, the quiescent state of a seed makes it more resistant than growing plants to environmental stresses such as cold and drought. On the other hand, the embryo inside the seed makes it prone to be injured by mutagens such as IR which can not be obstructed by quiescent state of seeds. The radiosensitivity of mammal embryos to X-ray exposures has been studied in the early days.\(^{39}\) Unlike low-LET radiation which equably penetrates through the materials, heavy ions cause significant damages on their trace inside the materials. Recently, Furusawa et al. revealed that heavy ion radiation acing on embryos of silkworm induced higher frequencies of somatic mutation than that acting on other tissue.\(^{57}\) For plants, embryo inside the seeds was also found to be more sensitive to IR than other tissues.\(^{39}\) Even when the charged partials can not reach the embryos, the damage effects can still be induced through long-distance bystander effects.\(^{40}\) Comparisons of radiosensitivities of plant seeds and seedlings were also reported by Zhang et al.\(^{5,28}\) in a study of micronucleus induction by neutrons and \(\gamma\)-ray, in which the ratio of cell with micronucleus in seed to that in seedling induced by \(\gamma\)-ray is about 10 times of neutrons. Their results strongly suggest that, compared with low LET-IR, high-LET IR is more effective in damage producing on plant seeds than seedlings. Hence, we speculate that after low dose exposures, more damages effects were found in the rice seeds than seedlings due to higher radiosensitivities of embryo inside the seeds. At higher doses in the present study, especially at 20 Gy, a significant higher level of chromosomal damages was observed in the seedlings than in the seeds. Significant lethal damages were also induced in the seedlings at the highest dose. These results suggest that high levels of chromosomal damages in seedlings at high dose may be a consequence of global apoptotic effects triggered by radiation stress.

Our observations of root apical meristem showed the existence of NMCC in interphase cell nucleus after carbon ion exposure (Fig. 3). At doses below 2 Gy, a significantly higher level of NMCC was observed in the dry and wet seeds than in the seedlings. Chromatin condensation was found to take place during both apoptosis and stress induced non-apoptotic damages.\(^{41-43}\) Stress induced non-apoptotic chromosome condensation has been shown to appear in the entire cell population without mitotic shutdown of metabolic activity or delayed death.\(^{43}\) It was speculated to be a checkpoint at which the cell reduced the putative DNA replication errors induced by stress before continues growing.\(^{42}\) Studies on germination of plant embryo have shown that chromatins in quiescent seed embryos is highly condensed.\(^{44}\) Hence, that radiation is inefficient in de-condensation during germination may be one of the reasons of abnormal chromosome condensations in the seed assay. In plant seeds, the action of cell is expansion rather than division at the early stage of germination. This character of plant seed makes it possible to distinguish the damage effect on cell expansion from that on cell division. As showed in Fig. 6, compositions of different types of MCAs induced by carbon ions were found in different rice materials. In the seedling assay, MCAs were mainly consisted of bridges, fragments, laggard chromosomes and disturbed spindles. Compared with the seedling assay, a higher percentage of sticky and uneven dispensed chromosomes were found in the dry and wet seed assays. The different composition of the types of MCAs between seeds and seedlings offers insights in the comparison between growing and non-growing tissues in responding to radiation stress. We argue that more laggard and disturbed chromosomes in the seedling assay implies that mitotic spindle damages are prone to appear in growing tissues. A relatively higher frequency of sticky and uneven dispersed chromosome in the seed assay indicates an involvement of damages of radiosensitive factors related to chromosome structure in response to radiation exposure. However, this hypothesis requires further investigations.

In this study, we present the results of carbon ion-induced biological effects including survival, root growth and mitotic activation, as well as chromosome aberration on rice. Our results demonstrated different radiosensitivities between rice seeds (dry and wet) and seedlings in multiple biological endpoints. Comparisons of the three rice materials provide a new insight into the mutagenetic effects of heavy
ion radiation at lower dose. Additionally, the different cellular and molecular responses to IR between quiescent seeds and growing seedlings suggest new processes in the production of radiation-induced new crops should be considered.

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