Energy-dependent RBE of Neutrons to Induce Micronuclei in Root-tip Cells of *Allium cepa* Onion Irradiated as Dry Dormant Seeds and Seedlings

WENYI ZHANG¹, KAZUO FUJIKAWA², SATORU ENDO³, MASAYORI ISHIKAWA¹, MEGU OHTAKI⁴, HIDE OIKEDA⁵, and MASAHARU HOSHI¹*

RBE/Neutron/Micronucleus/Onion.

The relative biological effectiveness (RBE) of various energy neutrons produced from a Schenkel-type accelerator at the Research Institute for Radiation Biology and Medicine, Hiroshima University (HIRRAC), compared with ⁶⁰Co gamma-ray radiation was determined. The neutron radiations and gamma-ray radiation produced good linear changes in the frequency of micronuclei induced in the root-tip cells of *Allium cepa* onion irradiated as dry dormant seeds (seed assay) and seedlings (seedling assay) with varying radiation doses. Therefore the RBE for radiation-induced micronuclei can be calculated as the ratio of the slopes of the fitted linear dose response for the neutron radiations and the ⁶⁰Co gamma-ray radiation. The RBE values by seed assay and seedling assay decreased to 174 ± 7, from 216 ± 9, and to 31.4 ± 1.0, from 45.3 ± 1.3 (one standard error), respectively, when neutron energies increased to 1.0 MeV, from 0.2 MeV, in the present study. Furthermore, the ratio of the micronucleus induction rates of seed assay to seedling assay by gamma-ray radiation was much lower than that by neutron radiations.

INTRODUCTION

The energy-dependent relative biological effectiveness (RBE) of neutrons plays a very important role in the planning of neutron therapy¹², in risk estimating, and in drawing safety standards for occupational and public populations⁸, and especially in reassessing cancer induction among the survivors of the Hiroshima and Nagasaki atomic bombings⁴–⁷. It is also of great interest in the better understanding of radiobiology⁸,⁹.

It was reported that for both cell lethality and neoplastic transformation in C3H 10T1/2 mouse cells neutron, RBE had a maximum value at neutron energy of about 0.35 MeV¹⁰–¹². The neutron RBE to induce initial DNA damages and chromosomal exchanges in human lymphocyte cells and to induce hprr mutation in Chinese hamster V79 cells reached peaks at neutron energies of approximately 0.37¹³ and 0.57¹⁴ MeV, respectively.

Concerning the RBE of various energy neutrons and the large neutron discrepancies in the Dosimetry System 1986 (DS86)¹⁵ of the Hiroshima dosimetry system between measurements and calculations for low-energy neutrons, we are very much interested in the neutrons with low energies that ranged from 0.2 to 1.0 MeV. The peaks of the neutron energy spectra are approximately 0.5 and 2 MeV in the Hiroshima and Nagasaki atomic bombings¹⁵, respectively. The radiation effects for A-bomb survivors, such as stable chromosomal aberration at a given dose in Hiroshima, are larger than in Nagasaki⁹.

In plants, however, the frequency of micronuclei induced in the root-tip cells of *Allium cepa* onion seedlings by ionizing radiation and chemicals is widely used as a simple measure of chromosome damage¹⁶–¹⁸. It was reported that the frequencies of micronuclei induced in the root-tip cells of *Allium cepa* onion exposed as seedlings to neutron-and-gamma-mixed radiations emitted from a ²⁵²Cf source at the Research Institute for Radiation Biology and Medicine, Hiroshima University, were about 16¹⁹ and 18²⁰ times higher, respectively, than the frequencies of those exposed to ⁶⁰Co gamma-ray radiation. The sensitivity of micronucleus induction in the root-tip cells of *Allium cepa* onion exposed as dry dormant seed to ²⁵²Cf neutrons is 150 times higher than that of those exposed to ⁶⁰Co gamma-ray radiation²¹. In the present study, we used the same method as the one in our previous experiment²¹. Therefore, by using this high sensitivity to neutron radiation compared to ⁶⁰Co gamma-ray radiation, we could obtain the RBE for radiation-induced micronuclei in the root-tip cells of *Allium cepa* onion seedlings exposed as dry dor-
mant seeds (seed assay) and seedlings (seedling assay), calculated as the ratio of the slopes for neutron radiations vs. 60Co gamma-ray radiation\(^{20, 21}\), using the methodology of micronucleus assay developed by Han moto\(^{22}\) in the present study.

MATERIALS AND METHODS

Radiation sources

Various energy neutrons were produced from a neutron irradiating system, the Hiroshima University Radiobiological Research Accelerator (HIRRAC), installed at Hiroshima University. HIRRAC is usually operated at a high proton beam current of 1 mA. With a Schenkel-type accelerator (HN-3000 BL, Nisshin High Voltage Co. Ltd., Japan), the protons are accelerated and bombard the \(^7\)Li target. The neutrons are produced with the \(^7\)Li(p, n)\(^7\)Be reaction\(^{23, 24}\). There are two beam lines in HIRRAC for neutron irradiations, a horizontal beam line (H-line) and a vertical one (V-line) using the \(^7\)Li target. The V-line is suitable to irradiating cells in a monolayer in medium\(^ {23}\). The doses of neutrons and gamma rays were measured at room temperature by using paired ionization chambers IC-17 and IC-17G (models 565-RTG and 561-RGG graphite), (Far West Technology, Inc., Goleta, CA, USA) at high voltage +250 V calibrated against the Japanese Radiological Physicists (JARP) tertiary standard chamber at Hiroshima University\(^ {23, 25}\). The chamber tissue-equivalent dose rates and dose contributions from accompanying gamma rays and other conditions used in the present study are given in Table 1.

A 60Co gamma-ray source (1.11 \times 10^{14} Bq on March 1, 1999, Shimadzu Seisakusho, Tokyo, Japan) installed at Hiroshima University was used for gamma irradiation\(^ {25}\). The tissue dose rate was approximately 44.6 Gy·h\(^{-1}\) at room temperature, as measured by the JARP chamber at a distance of 80 cm from the source, where the tubes containing samples were placed under a 4 \times 300 \times 300 mm square Lucite plate (1.2 g·cm\(^{-2}\)).

Dry dormant onion seeds and seedlings

Onion (Allium cepa L.) seeds of variety OK Yellow that had been harvested in July 1999 were kindly supplied by Dr. Yoshi-hiko Yonezawa, Naruto University of Education, Naruto, Japan. The seeds were stored in a refrigerator at 4°C under dry conditions until use.

Before irradiation for seed assay, the seeds were first filled into 12 mm (diameter) \times 42 mm polystyrene tubes (Asahi Techno Glass Corporation, Chiba, Japan), then subjected to irradiation. For seedling assay, the seeds were first sown on paper beds soaked with distilled water in 60 mm (diameter) \times 15 mm IWAKI tissue-culture dishes and allowed to germinate on the beds at 25°C. At 48 h after sowing, the resultant seedlings with roots of approximately 5 mm were quickly sampled and transferred on freshly prepared distilled water soaked paper beds in 35 mm (diameter) \times 13 mm IWAKI tissue-culture dishes and immediately subjected to irradiations.

The main element compositions of dry dormant seeds and seedlings and their weight percent are given in Table 2.

Timing for micronucleus assays

For seed assay, one to two days after irradiations the irradiated seeds were sown on paper beds soaked with distilled water in 35 mm IWAKI tissue-culture dishes and allowed to germinate on the beds at 25°C. Then 72 h after sowing, the resultant seedlings with roots of about 1 cm were sampled and subjected to micronucleus assays. For seedling assay, the irradiated seedlings were cultured at 25°C in the same dishes for 24 h before being subjected to micronucleus assays.

The sampling time was determined based on the observations made in our previous experiment\(^ {21}\). The mitotic index (number of mitotic cells per 100 meristematic cells) and the frequency of micronuclei (number of micronuclei per 100 interphase cells) were measured in the root-tip cells of onions irradiated as dry dormant seeds with 3.5 Gy of 252Cf mixed radiation or 196 Gy of 60Co gamma rays at 24-h intervals from 48 to 120 h after sowing. In both the irradiated samples and in the controls, the mitotic indexes were low at 48 h after sowing and increased to a normal or subnormal level at 72 h; thereafter they retained a high level. The frequencies of micronuclei in both irradiated samples increased from the control level to a high level of 48 to 72 h after sowing; they retained the high level at 96 and 120 h. These

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**Table 1.** Doses applied to seeds and seedlings, and dose rates, dose contributions from accompanying gamma rays, and other conditions used to produce various energy neutrons from HIRRAC in the present study.

<table>
<thead>
<tr>
<th>Irradiation</th>
<th>Neutron energy (MeV)</th>
<th>Applied dose(^ {a}) (Gy)</th>
<th>Target</th>
<th>Beam line</th>
<th>Distance(^ {b}) (cm)</th>
<th>Filter</th>
<th>Dose rate(^ {a}) (cGy·min(^{-1}))</th>
<th>Dose contribution of gamma rays(^ {a}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seed</td>
<td>0.2</td>
<td>1–4</td>
<td>(^7)Li</td>
<td>V</td>
<td>10</td>
<td>no</td>
<td>4.8–12.3</td>
<td>4.8–6.9</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>26.7–75.5</td>
<td>0.9–3.4</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19.2–51.3</td>
<td>1.8–6.7</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20.4–25.0</td>
<td>2.0–8.6</td>
</tr>
<tr>
<td>Seedling</td>
<td>0.2</td>
<td>0.05–0.2</td>
<td>(^7)Li</td>
<td>V</td>
<td>10</td>
<td>no</td>
<td>4.7–15.1</td>
<td>4.4–6.9</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20.2–39.1</td>
<td>0.9–3.4</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>18.7–24.7</td>
<td>1.8–6.7</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>19.2–28.7</td>
<td>2.0–8.6</td>
</tr>
</tbody>
</table>

\(^{a}\)Doses and dose rates of tissue-equivalent material in chambers. \(^{b}\)Source to samples.
results also confirmed the previous reports that in the root-tip cells of onion seedlings, the mitotic division onsets some 48 h after sowing and the cell cycle duration are about 21 h\(^{16,26,27}\). In other words, the micronuclei scored in the roots of treated dry dormant seeds were produced through the first mitotic cycle after irradiations.

**Micronucleus assay**

The roots of sampled seedlings were fixed, macerated, and stained for 15 minutes with a solution containing acetic dahlia and 1 N HCl at a volume ratio of 7:3\(^{22}\), which was prepared by dissolving a 0.5 g sample of dahlia violet (Wako Pure Chemical Industries, Ltd., Osaka, Japan) into 100 ml of 30% acetic acid. The treated roots were then briefly washed with distilled water for 5–10 min, and the terminal 1–2 mm of the roots were mounted by using 50% glycerin as a medium and squashed on slide glasses, one root to each glass. The semipermanent preparations made in this way were microscopically inspected at a magnification of 400\(^{22}\) for the presence of more than one nucleus in the meristematic cells at the interphase. The additional nuclei, which were smaller than the normal, were scored as micronuclei\(^{28}\). The frequency of micronuclei was determined for each irradiation as the unweighted mean for 5 slides, on each of which about 1,000 interphase cells were observed.

**Absorbed doses in dry dormant onion seeds and seedlings**

The doses applied to seeds and seedlings in the present study were also given in Table 1. Briefly, given by the paired chambers, the doses applied to seeds were 1, 2, 3, and 4 Gy of neutrons, or 25, 50, 70, and 100 Gy of \(^{60}\)Co gamma rays; the doses applied to seedlings were 0.05, 0.1, 0.15, and 0.2 Gy of neutrons, or 0.25, 0.5, 0.7, and 1 Gy of \(^{60}\)Co gamma rays.

To convert all absorbed doses measured by the paired chambers in terms of Gy in tissue-equivalent (TE) to the doses absorbed in onion seed and seedling tissues (OS), the kerma coefficients of neutrons and gamma rays in onion seed and seedling tissues \((k_{OS})\) were calculated by

\[
k_{OS} = \sum_{i} k_i E w_i,
\]

where \(k_i\) is the kerma coefficient of radiation with energy \(E\) for element \(i\), and \(w_i\) is the weight percent of element \(i\) in dry dormant onion seeds and seedlings (Table 2). The \(k_i\) values for various energy neutrons were taken from ICRU Report 26\(^{29}\); the values for gamma rays were taken from a report by Hubbell\(^{30}\). Similarly, the \(k_{TE}\) values for neutrons and gamma rays were calculated by using the element compositions of tissue-equivalent reported by ICRU Report 26\(^{29}\) and ICRU Report 33\(^{31}\), respectively. The kerma ratios of \(k_{OS}\) to \(k_{TE}\) can be used as conversion factors of tissue-equivalent doses in paired chambers to absorbed doses in onion seeds and seedlings. The kerma coefficients and kerma ratio values of various energy neutrons and \(^{60}\)Co gamma rays for dry dormant onion seeds, seedlings, and tissue-equivalent materials used in the paired chambers are given in Table 3.

**Maximum likelihood method with a Poisson-response model to estimate RBE values**

Let the data be described by \([y_1, n_1, D_1, z_1] [y_2, n_2, D_2, z_2], \ldots, [y_k, n_k, D_k, z_k]\), where \(n_i\) denotes the number of observed cells, \(y_i\) the number of micro-

<table>
<thead>
<tr>
<th>Table 2. Primary element compositions of dry onion seeds and seedlings.</th>
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<tbody>
<tr>
<td><strong>Element</strong></td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>Seed</td>
</tr>
<tr>
<td>Hydrogen</td>
</tr>
<tr>
<td>Carbon</td>
</tr>
<tr>
<td>Nitrogen</td>
</tr>
<tr>
<td>Oxygen</td>
</tr>
<tr>
<td>Sodium</td>
</tr>
<tr>
<td>Magnesium</td>
</tr>
<tr>
<td>Silicon</td>
</tr>
<tr>
<td>Phosphorus</td>
</tr>
<tr>
<td>Sulfur</td>
</tr>
<tr>
<td>Chlorine</td>
</tr>
<tr>
<td>Potassium</td>
</tr>
<tr>
<td>Calcium</td>
</tr>
<tr>
<td>Iron</td>
</tr>
</tbody>
</table>

\(^{a}\) Analyzed by Kawatetsu Techno Research Company, Chiba 260-0835, Japan.

| Table 3. Kerma coefficients and kerma ratios of \(^{60}\)Co gamma ray, \(^{252}\)Cf neutrons, and various energy neutrons from a 3 MV accelerator at Hiroshima University (HIRRAC) in onion seed and seedling tissues and tissue-equivalent materials in the paired chambers. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Radiation** | **Energy (MeV)** | **Kerma coefficients\(^{b}\) \((\times 10^{-13} \text{ Gy m}^2)\)** | **Kerma ratio\(^{c}\)** |
| | | Seed | Seedling | Tissue equivalent | Seed | Seedling |
| Neutron | 0.2 | 0.778 | 1.036 | 0.989 | 0.787 | 1.048 |
| | 0.5 | 1.254 | 1.649 | 1.580 | 0.794 | 1.044 |
| | 0.8 | 1.581 | 2.081 | 1.985 | 0.797 | 1.048 |
| | 1.0 | 1.864 | 2.557 | 2.354 | 0.792 | 1.086 |
| \(^{60}\)Co gamma ray | 1.25\(^d\) | 0.573 | 0.588 | 0.588 | 0.974 | 1.000 |

\(^{b}\) Obtained from ICUR Report 26\(^{29}\) and the report by Hubbell\(^{30}\) for neutrons and gamma rays, respectively. \(^{c}\) Calculated by the element compositions given in Table 2, using equation 1 in text, respectively. \(^{d}\) Kerma coefficients of seed and seedling tissues to those of tissue-equivalent materials in chambers. \(^{d}\) Averaged for gamma rays emitted from the \(^{60}\)Co source.
nuclei, \( D \) is the exposed dose, and \( z_i \) denotes the indicator variable which has the values of

\[
z_i = \begin{cases} 1, & \text{in case of neutron exposure}, \\ 0, & \text{in case of gamma ray exposure}, \end{cases}
\]

for the \( i \)th cite. Then the likelihood function that we adopted can be expressed as

\[
L(\theta) = \prod_{i=1}^{k} \frac{[n_i(1 + RBE \cdot \beta D_i)]^{y_i}}{y_i!} e^{-n_i(1 + RBE \cdot \beta D_i)},
\]

where \( \beta \) and \( RBE \) are parameters.

Maximizing the log likelihood function \( \log L(\theta) \) with respect to the parameters \( \beta \) and \( RBE \), we learned their maximum likelihood estimates. The magnitudes of their standard errors were also estimated by using a score method based on Fisher’s Information Matrix.

**RESULTS**

*Frequencies of micronuclei induced by neutrons and gamma rays*

In Figs. 1 and 2, the frequencies of micronuclei \( F (%) \) induced in the root-tip cells of onion seedlings after exposures as dry dormant seeds and seedlings to various energy neutrons and \( ^{60} \text{Co} \) gamma rays are plotted against the absorbed doses in onion seed and seedling tissues \( D (\text{Gy}) \), respectively.

Using a maximum likelihood method with a Poisson-response model described above, we fitted the dose-response data to linear equations of

\[
F = (0.13 \pm 0.01) + bD \quad (2a)
\]

and

\[
F = (0.14 \pm 0.02) + bD \quad (2b)
\]

for seed assay and seedling assay, respectively. The numerical terms in the equations above are the spontaneous frequencies of micronuclei (with one standard error), determined as averages of concurrent controls from six experimental runs for seed assay and five experimental runs for seedling assay. The \( b \) values, that is, the slopes of fitted dose-response linear equations, are the numbers of micronuclei induced in 100 interphase root-tip cells of onion seedlings per Gy. Calculated \( b \) values as regression coefficients for various energy neutrons and \( ^{60} \text{Co} \) gamma ray by seed assays and seedling assays are given in Table 4 as the induced rates of micronuclei.

**RBE values**

The RBE is defined, for a specific radiation, as the ratio of dose of reference radiation required to produce a specific level of response to a dose of specific radiation required to produce an equal response\(^{25}\). In the present study, parameter \( b \) (that is, the slope of the fitted linear dose-response Eq. 2, \( a \) and \( b \)) is the number of micronuclei induced in 100 interphase cells per Gy given by neutrons and gamma rays. That is, \( b^{-1} \) presents the dose required to induce one micronucleus in 100 interphase cells. Therefore the RBE values of various energy neutrons to induce micronuclei in the root-tip cells of onion seedlings after exposures as dry dormant seeds and seedlings could be calculated as the ratio of \( b_n \) for various energy neutrons to \( b_g \) for \( ^{60} \text{Co} \) gamma ray\(^{21,28,33,34} \). The RBE values for various energy neutrons are listed in Table 4.

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**Fig. 1.** The frequencies of micronuclei observed in the root-tip cells of onion seedlings exposed as dry dormant seeds plotted against the absorbed doses (Gy) in onion-seed tissues given by 0.2 (open squares), 0.5 (solid diamonds), 0.8 (solid triangles), and 1.0 (inverted solid triangles) MeV neutrons (the top axis) and \( ^{60} \text{Co} \) gamma rays (open circles, the bottom axis). The solid squares represent the spontaneous frequency of micronuclei. The fitted lines are plotted by the use of equation 2a in text.

**Fig. 2.** The frequencies of micronuclei observed in the root-tip cells of onion seedlings exposed as seedlings plotted against the absorbed doses (Gy) in onion seedling tissues given by 0.2 (open squares), 0.5 (solid diamonds), 0.8 (solid triangles), and 1.0 (inverted solid triangles) MeV neutrons (top axis) and \( ^{60} \text{Co} \) gamma rays (open circles, bottom axis). The solid squares represent the spontaneous frequency of micronuclei. The fitted lines are plotted by the use of equation 2b in text.
In Fig. 3, the RBE values of neutrons are plotted against the neutron energies (MeV). As seen in Fig. 3 and Table 4, when the neutron energies increase to 1.0 MeV, from 0.2, the neutron RBE values decrease to 174 ± 7, from 216 ± 9, and to 31.4 ± 1.0, from 45.3 ± 1.3, for seed assay and seedling assay, respectively.

**DISCUSSION**

Table 4 gives the sensitivities of the dry onion seeds and seedlings to neutrons with energies ranging from 0.2 to 1.0 MeV. It also shows the neutrons emitted from a $^{252}$Cf source and $^{60}$Co gamma rays for producing micronuclei in the root-tip cells of onion seedlings after irradiation as dry dormant seeds and seedlings. For gamma rays, the induced rate of micronuclei, $b_g$, of seeds was about 1% of seedlings; but for neutrons, the induced rate of micronuclei, $b_n$, of seeds was about 4% of seedlings. In other words, the ratio of the induction rate of micronuclei in the root-tip cells of *Allium cepa* onion seedlings exposed to $^{60}$Co gamma rays was about 5 times higher than that to neutrons, given that the RBE values of dry dormant seeds were about 5 times higher than those of the seedlings. This is because a large difference in oxygen content in seeds and seedlings greatly influences the degree of response, especially to low-LET radiations. As seen in Table 4, since oxygen can greatly enhance the effectiveness of low-LET radiations (such as gamma rays) compared to high-LET radiations (such as fast neutrons), its presence during irradiation (irradiation as seedlings) results in lower RBE values than its absence during irradiation (irradiation as dry dormant seeds) does$^{32,39}$. For example, for a single locus somatic mutation in maize, it was reported that an RBE of fission neutrons versus 250 kVp X rays of 67 was given for seeds of 6.7% moisture, whereas after soaking for 36 hours before irradiation, the RBE was reduced to five$^{32}$. In other words, this lower RBE
of seedlings resulted from an increased effectiveness of low-LET radiations. For the same maize system mentioned above, under conditions of oxygen exclusion, the RBE for fission neutrons versus gamma rays was more than 100\cite{32}. Another reason is more likely that the irradiated cells in seedlings might prefer cell death to repair. In this situation, as the damaged cells die, the incidence of micronuclei becomes low (for neutron irradiations, the efficiency to induce micronuclei in seeds was about 4% of that in seedlings; for gamma rays, about 1%).

From Table 4, when neutron energies increased to 1.0 MeV, from 0.2, the induced rates of micronuclei in 100 interphase cells per Gy by neutrons decreased to 3.64, from 7.55, for seeds and to 111, from 160, for seedlings\cite{15,12}. This is because for given types of radiations, the higher the energy, the lower its biological effectiveness\cite{12}. Moreover, both for seeds and seedlings the micronucleus induction rates in neutron-treated samples were much higher than those in gamma-ray treated samples. This was due to the difference in LET values between $^{60}$Co gamma rays (LET = 0.2 keV/µm) and neutrons (for neutrons emitted from $^{252}$Cf source, the average LET is 80–100 keV/µm).

Although the RBE values of neutrons to induce micronuclei in the root-tip cells of onion seedlings exposed as dry dormant seeds are much higher than those exposed as seedlings, it is very interesting to note, from Fig. 3, that the changes of neutron RBE values with neutron energies ranging from 0.2 to 2.1\cite{20,21} MeV are similar; that is, the neutron RBE values decrease significantly with increasing neutron energy.

In summary: (i) the neutron radiations and the gamma-ray radiations both produced approximate linear changes in the frequency of micronuclei induced in the root-tip cells of Allium cepa onion irradiated as either dry dormant seeds or seedlings with varying radiation doses; (ii) the rate of micronucleus induction by neutrons in the root-tip cells of Allium cepa onion seedlings was much higher than that by $^{60}$Co gamma rays; (iii) the RBE values of neutrons to induce micronuclei in the root-tip cells of Allium cepa onion seedlings exposed as dry dormant seeds and seedlings both decreased to 150 ± 6, from 216 ± 9,\cite{21} and to 18 ± 5, from 216 ± 9\cite{20}, respectively, when the neutron energies increased to 2.1, from 0.2,\cite{20,21} MeV.

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