



RESEARCH ARTICLE

# *In vitro* acute gamma radiation on tissue of pink and white lotus (*Nelumbo nucifera* Gaertn.) in Thailand

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## Abstract

This research aimed to investigate the age of embryos of pink and white lotus for *in vitro* propagation and examine the sensitivity of young lotus plants derived from tissue culture to acute gamma radiation. The seeds of both lotus varieties were 4-week, 6-week and 8-week-old embryos cultivated on solid MS medium. They were then transferred to solid MS medium supplemented with 3 mg/L BA. After culturing, the 6-week-old pink lotus embryos had an average maximum petiole length of  $16.78 \pm 1.01$  cm and an average of  $11.84 \pm 0.44$  roots/explant, while the 6-week-old white lotus embryos had an average maximum petiole length of  $15.48 \pm 0.68$  cm and an average of  $11.76 \pm 0.70$  roots/explant. Each young lotus plant produced one shoot. After treating the young lotus plants with liquid MS medium supplemented with 3 mg/L BA, both lotus varieties showed increased shoot numbers at  $3.54 \pm 0.13$  and  $3.40 \pm 0.91$  shoots/explant. Young lotus plants of both varieties were exposed to gamma radiation levels of 0, 20, 30 and 40 Gy. The LD<sub>50</sub> value for pink lotus was 26.138 Gy, while the LD<sub>50</sub> value for the white lotus was 32.031 Gy. The LD<sub>50</sub> value will offer vital information for determining the optimal dosage of gamma radiation for both pink and white lotus *in vitro*.

## Keywords

Acute gamma radiation; Embryo; LD<sub>50</sub>; Plant tissue culture; Pink lotus; White lotus

## Introduction

The lotus (*Nelumbo nucifera* Gaertn.) is widespread across Asia, the Northern sections of Oceania, parts of South America and the Eastern, Southern and Northern regions of North America (1). In Thailand, there are approximately 1500 rai of lotus production areas spread throughout every region of the country (2). The lotus is a perennial aquatic plant found in lakes, marshes and ponds. It is considered an economically important aquatic plant in Thailand because every part can be utilized. For example, the stems and rhizomes are used as food ingredients, young and mature leaves as well as the flowers are used for blood nourishment and the pollen is used to make a beneficial tea for heart health. Lotus seeds are used as a remedy for treating heat-related conditions (3) and also contain bioactive compounds such as alkaloids and flavonoids with antioxidants, antipyretic, anti-cancer and antiviral properties (4). In Thailand, pink and white lotus plant varieties are priced at 80-200 Baht, with rare or new varieties with different colors fetch-

ing up to 5-10 times more. However, the production of new lotus varieties remains insufficient to meet market demand because the conventional method of lotus propagation uses rhizomes and takes a long time. The majority of lotus is spread by underground branches, typically from the extended rhizome. Continuous vegetative multiplication from the rhizomes or shoots might result in cultivar degeneration, which is another issue (5). Moreover, most hybrid lotus seeds obtained from cross-pollination are weak and often die prematurely (6).

There are few studies reporting the lotus plant's *in vitro* culture. Cultivation of young shoot tips and leaves through tissue culture has a low survival rate due to high microbial contamination. Plant parts obtained from parent plants grown underwater have a higher contamination rate than those grown above the water surface (5). The hindrance of *in vitro* seed germination often arises when plants are collected from the open field and stored under unfavorable conditions, necessitating the development of a more effective surface sterilization protocol (7). Therefore, using tissue culture from explants of embryonic lotus has become a viable option to address this issue (8). Previous reports have documented somatic embryogenesis from buds of lotus and shoot apical meristems from the buds (4). Calluses obtained from immature cotyledons and embryos often exhibit good growth (9). Improving plant varieties through tissue culture is a technique that is free from pathogens, reduces space requirements and minimizes production costs. Plant tissue culture also enables the rapid multiplication of plant stocks and preserves authentic genetic material, thereby enhancing genetic diversity with potential benefits for various future aspects including population genetic engineering (10).

Acute gamma radiation is a popular method to induce plant breeding mutations because it is straightforward, cost-effective and does not leave any residual radiation in the planters (11). When combined with plant tissue culture techniques, this method can effectively replace conventional cross-breeding since it requires less time for suitable gamma radiation levels to induce mutations in different plant species. The LD<sub>30</sub> - LD<sub>50</sub> values vary depending on the botanical characteristics and the radiation exposure format. Radiation affects cell division and metabolism, particularly with regard to the genetic materials controlling various plant traits. When genetic materials undergo changes, this can result in alterations of phenotypic traits such as flower color, leaf color, flower shape, leaf shape and plant height (11, 12). It was reported that the LD<sub>50</sub> for germination rates of irradiated rhizomes was determined to be 12 Gy (13). Morphological traits of lotus plants grown from these irradiated rhizomes exhibited variations compared to those of the original lotus plants. The utilization of mutant varieties to develop new commercial cultivars remains essential. However, it is imperative to continue planting, testing and selecting plants with high stability and desired characteristics for both the market and farmers. There are reports indicating that irradiating the stem or callus can enhance the probability of mutations (14, 15). It was reported that variations in the gamma

radiation dosage administered to different plant organs, noting that *Citrus* spp. seed irradiation (LD<sub>50</sub> of 127 Gy) requires doses several times higher than those used for nodal segments (LD<sub>50</sub> of 25 Gy) (16). The gamma dose should be carefully selected to induce a higher mutation rate in the targeted trait while minimizing its impact on the overall genetic background. Therefore, this study was conducted to increase the quantities of pink and white lotus seedling using plant tissue culture techniques from embryos and then subjecting the cultures to acute gamma radiation. The first step was to induce multiple shoots by studying variety and embryo age. The second step was to calculate the LD<sub>50</sub> after gamma radiation. This process induces mutations and can serve as a direction for future lotus breeding as well as determining the appropriate gamma radiation levels for developing new lotus varieties, thereby adding value and potential for exporting aquatic plant products and creating income for future lotus cultivators.

## Materials and Methods

### Sample collection

Plant samples used in this experiment were pink and white lotus seeds (*Nelumbo nucifera* Gaertn.) belonging to the family Nelumbonaceae, gathered from the Nakhon Pathom Province, Thailand. The seeds of both lotus varieties were obtained from pods at 4, 6 and 8 weeks after pollination and the collected site coordinates were 13°48'30.1"N 100°16'24.4"E. The samples were cultured *in vitro* at the National Science and Technology Development Agency, Thailand.

### Obtaining germ-free embryos from pink and white lotus seeds

Lotus seeds aged 4, 6 and 8 weeks were dehusked and the outer green layers were removed. Then, the seeds were placed in 8-ounce culture tubes that had been sterilized by autoclaving and treated with 70% (v/v) ethyl alcohol for 1 min before discarding the alcohol. The 8-week-old lotus seeds were further soaked in sterilized distilled water and shaken at 150 revolutions per min (rpm) for one week.

Next, the lotus seeds were immersed in 95%(v/v) ethyl alcohol and sterilized by burning fire (repeated twice). The seeds were split lengthwise without damaging the embryos, which were then placed on solid MS medium, 30 g sucrose, 2.75 g Kelcogel®, adjusted pH to 5.6 without plant growth regulators for tissue culture. The tissue culture was carried out for one week at 25 ± 2 °C with light intensity of 65 μmol/m<sup>2</sup>/s<sup>-1</sup> for 16 h per day using fluorescent white light.

### Shoots induced directly from mature embryos *in vitro*

After 4 weeks of culture on solid MS medium (17), the embryos were sub-cultured under a tissue culture laminar flow using liquid MS medium supplemented with 3 mg/L BA at a 1:1 ratio with solid medium (8). The tissue culture was continued for another 4 weeks at 25 ± 2 °C with a light intensity of 65 μmol/m<sup>2</sup>/s<sup>-1</sup> for 16 h per day using fluorescent white light.

### Checking the radiosensitivity of pink and white lotus shoots *in vitro*

Healthy young lotus shoots (age 8 weeks from *in vitro*) were selected from both varieties and their leaves and roots were trimmed, leaving only the stems and apical buds. The shoots were placed in 8 ounce empty test tubes and exposed to gamma radiation doses of 0, 20, 30 and 40 Gy. The gamma radiation source was cobalt -60 (at a dose rate of 75 Gy/h, JS 8900 IR-155) and consisted of 6 rods provided by Paul Stephens Consultancy Ltd. at the Thailand Institute of Nuclear Technology (Public Organization) in Ongkharak District, Nakhon Nayok Province, Thailand. After irradiation, the plant segments were cultured on solid MS liquid supplemented with 3 mg/L of BA at a 1:1 ratio with solid medium and kept under 50% shade for one week. The tissue culture was then continued for another 4 weeks at  $25 \pm 2$  °C with a light intensity of  $65 \mu\text{mol}/\text{m}^2/\text{s}^{-1}$  for 16 h per day using fluorescent white light. The objective was to determine the gamma radiation dosage that led to calculate the lethal dose ( $\text{LD}_{50}$ ) (50% lethal dose at 4 weeks after irradiation) using the Probit method and observe the morphological characteristics and growth of both pink and white lotus shoots after gamma radiation exposure.

### Seedling rearing techniques for pink and white lotus

After exposure to gamma radiation for 8 weeks, the seedlings were washed to remove the gel adhering to the roots. Subsequently, the seedlings were placed in containers filled with sterilized water and kept at  $25 \pm 2$  °C under 16 h of light per day for one week. The seedlings were then transplanted into plastic containers filled with potting soil, using a quarter of the pot's capacity and kept in a greenhouse. Survival rates were recorded (5).

### Data analysis

Statistical data analysis was performed using SPSS version 24.0 with a significance level of  $p < 0.01$ .

- 1) Factorial design was performed to study the effects of embryo ages and lotus varieties on petiole length, root number and shoot number. The experiment was repeated 5 times. Pairwise mean differences were compared using the Scheffe's method.
- 2) The relationship between radiation dosage and seedling mortality rate was examined. The experiment was repeated 5 times and the  $\text{LD}_{50}$  value was calculated using the Probit method.
- 3) % of survival = (Number of seedlings after gamma radiation exposure/Total number of seedlings)  $\times$  100

## Results and Discussion

### Obtaining germ-free embryos from pink and white lotus seeds

Microbial contamination in pink and white lotus seeds aged 4 and 6 weeks after fertilization was eliminated by burning, with a 100% survival rate observed after culture on solid MS medium for one week. Microbial contamination in pink and white lotus seeds aged 8 weeks after fertilization was eliminated by burning and soaking them

for one week in distilled water, which had been boiled to disinfect the contaminants. However, this failed to soften the young seeds, which could not be cut open.

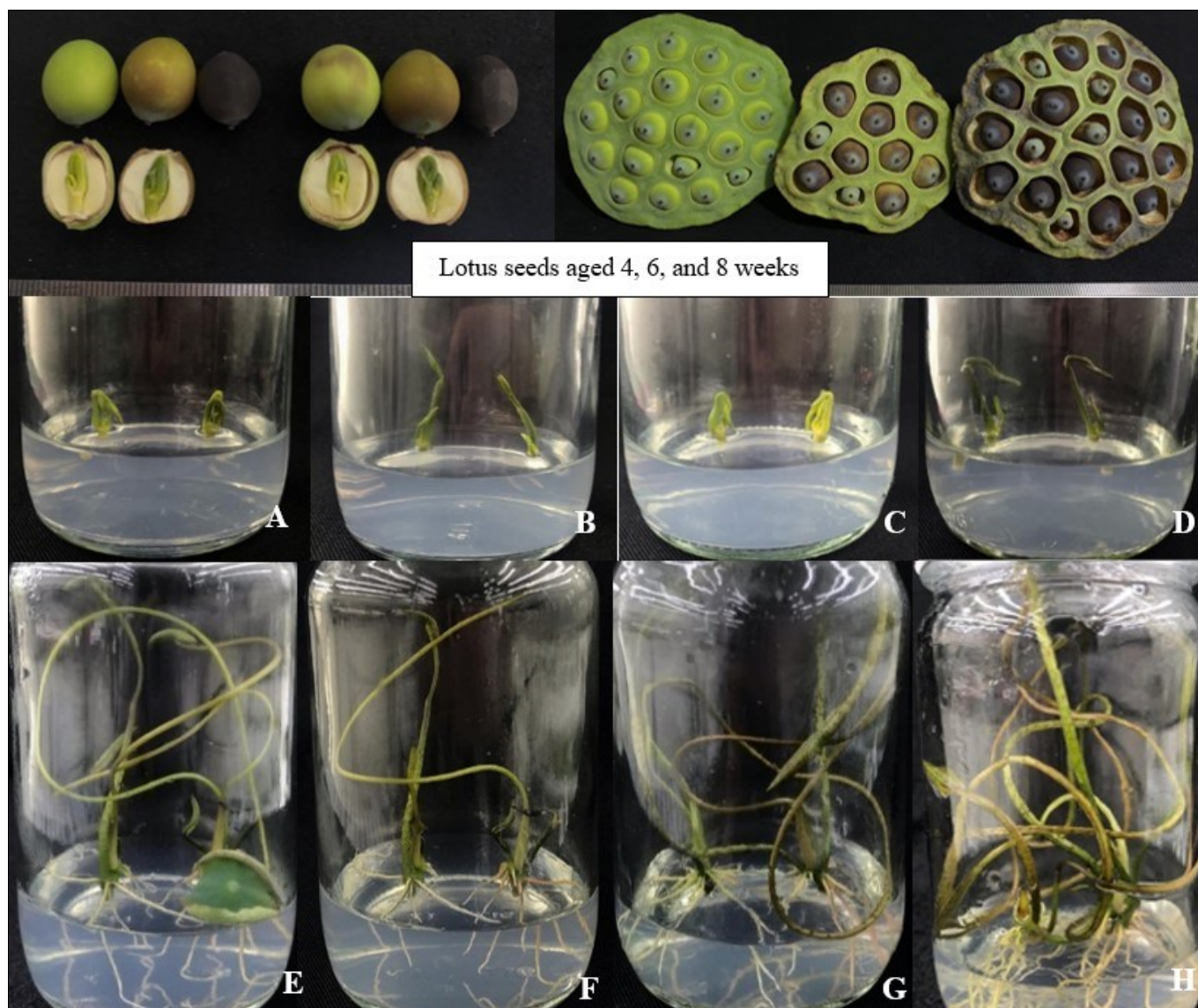
Mercuric chloride, one of the most hazardous chemicals utilized for sterilization, is extremely toxic. Ethanol, sodium hypochlorite and calcium hypochlorite are the most frequently employed agents for sterilization of plant materials, including seeds (7) and should be used in combination, which necessitates careful consideration of factors such as the specific type, concentration and duration of exposure (18). The process of making lotus seeds germ-free through burning also eliminated contamination by bacteria and fungi and the tissue characteristics were able to grow normally. These results demonstrated that the burning method was as effective as using chemical treatments to disinfect the contaminants. Reports are on the experiment to render the seeds of *Tupistra albiflora* K. Larsen germ-free using both chemical treatments and burning methods, with the burning method was found to be the most effective (19). Another study stated that plant seeds are organs and more robust and durable than other components such as the embryo and cotyledons (20). These components have high resistance to contamination, making them suitable as starting materials for tissue culture. However, in the case of pink and white lotus seeds aged 8 weeks, the seed coat structure hindered water and air penetration (Fig. 1). Consequently, these mature seeds could not be dissected to obtain explants for tissue culture (21). To overcome this limitation, chemical or mechanical methods may be employed to remove the tough seed coat. The mechanical approach is time-consuming, while the chemical method often involves using sulfuric acid to soften the hard seed coat (20). Chemical treatment can also potentially harm the embryo and inhibit shoot development.

Therefore, burning lotus seeds to eliminate contamination from spreading germs proved to be a time and chemical-efficient alternative. Typically, several methods exist for disinfecting lotus parts before tissue culture. These methods often involve a combination of chemicals and various techniques (9, 22, 23). However, sterilizing lotus seeds by burning is a simple and quick method and no contamination was detected. Burning fire has limitations when it comes to choosing it as a method of application. This is because the heat generated by fire can damage plant tissue, making it more suitable for organs or tissues that possess a thick or robust covering.

### Shoots induced directly from mature embryos *in vitro*

Results of shoot growth of pink lotus embryos aged 4 and 6 weeks after pollination and cultured for 4 weeks *in vitro* showed that these age groups had an average maximum petiole length of  $16.78 \pm 1.01$  cm and  $10.48 \pm 0.51$  cm respectively, while the average maximum number of roots found was  $11.84 \pm 0.44$  and the average minimum was  $4.24 \pm 0.38$ . For white lotus embryos, the average maximum petiole length was  $15.48 \pm 0.68$  cm and the average minimum was  $6.5 \pm 1.20$  cm, with the average maximum number of roots  $11.76 \pm 0.70$  and the average minimum





**Fig. 1.** Growth of pink and white lotus embryos cultured on MS medium after one week (A-D) and 4 weeks (E-H), (A, B) Pink lotus embryos at 4 and 6 weeks after pollination (E, F) Lotus seedlings after 4 weeks of culture, (C, D) White lotus embryos at 4 and 6 weeks after pollination (G, H) Lotus seedlings after 4 weeks of culture.

$4.92 \pm 0.67$  (Table 1). Each plantlet showed only one shoot. When comparing the experimental results, the 6-week-old embryos of both pink and white lotus had higher average petiole lengths and greater number of roots compared to the 4-week-old embryos. These findings indicated that the age of the embryos significantly affected the average petiole length and root numbers at a 99% confidence level.

However, the age of the embryos did not have a significant effect on shoot length and root number (Table 1). Shoots induced directly from mature embryos *in vitro* section, as aseptic seedlings are recommended as a source of explants in tissue culture investigations due to the reduction in the frequency of *in vitro* germination caused by seed dormancy (24).

**Table 1.** The petiole length, root and shoot number of pink and white lotus seedlings obtained from embryo tissue culture at 4 and 6 weeks after pollination on MS medium for 4 weeks.

Embryo age / After pollination	Pink lotus			White lotus		
	Petiole length	No. of roots/ explant	No. of shoots/ explant	Petiole length	No. of roots/ explant	No. of shoots/ explant
4 weeks	$10.48 \pm 0.51$	$4.24 \pm 0.38$	$1.00 \pm 0.00$	$6.50 \pm 1.20$	$4.92 \pm 0.67$	$1.00 \pm 0.00$
6 weeks	$16.78 \pm 1.01$	$11.84 \pm 0.44$	$1.00 \pm 0.00$	$15.48 \pm 0.68$	$11.76 \pm 0.70$	$1.00 \pm 0.00$
Embryo age (A)	**	**	ns	**	**	ns
Variety (B)	ns	ns	ns	ns	ns	ns
A × B	**	**	ns	**	**	ns

The values are presented as mean  $\pm$  standard deviation. \*\* indicates statistically significant differences at a confidence level of  $p < 0.01$ , while **ns** indicates no

After culturing pink and white lotus embryos on solid MS medium for 4 weeks and subsequently transferring them to liquid MS medium supplemented with 3 mg/L BA, the survival rate was observed. Among 50 pink lotus embryos, 82% survived after one week in the liquid medium, while among 50 white lotus embryos, 74% survived. No contamination of bacteria and fungi was found. At week 6, clear shoot growth of the lotus seedlings was observed. By week 8, the 4 week-old pink lotus embryos had an average maximum count of  $3.12 \pm 0.17$  shoots per explant, while white lotus embryos had an average maximum count of  $3.04 \pm 0.84$  shoots per explant. The 6 week-old pink lotus embryos had an average maximum count of  $3.54 \pm 0.13$  shoots per explant, while the white lotus embryos had an average maximum count of  $3.40 \pm 0.91$  shoots per explant. No statistically significant differences were found in shoot numbers between the 2 lotus varieties and embryo ages (Table 2).

**Table 2.** Number of shoots per explant of pink and white lotus seedlings obtained from embryo tissue culture at 4 and 6 weeks after pollination on MS medium for 4 weeks.

Embryo age	Pink lotus	White lotus
	No. of shoots/explant	No. of shoots/explant
4 weeks	$3.12 \pm 0.17$	$3.04 \pm 0.84$
6 weeks	$3.54 \pm 0.13$	$3.40 \pm 0.91$
Embryo age (A)	ns	ns
Variety (B)	ns	ns
A × B	ns	ns

ns indicates no statistically significant difference at a confidence level of

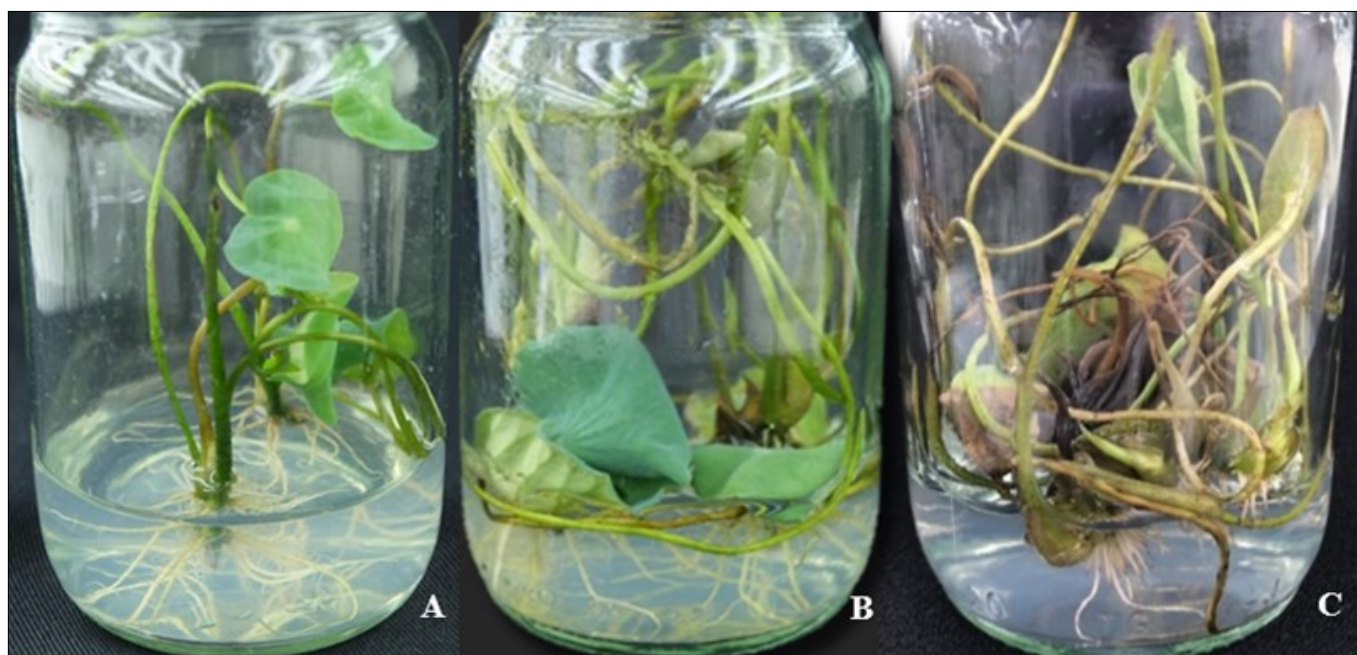
Consistent with earlier reports, tissue culture of lotus embryos on MS medium supplemented with 3 mg/L BA showed a statistically significant increase in average number of shoots compared to the control group without growth-promoting substances (5, 8). Increased shoot formation was attributed to the cytokinin BA which stimu-

lated the formation of new shoots by promoting shoot development, inducing new shoot growth and facilitating cell division (25-27). Young pink and white lotus seedlings cultured in liquid MS medium supplemented with 3 mg/L BA underwent a change in the color of their root tissues and petiole from white to black. Over time, the tissues gradually dried out and no new growth occurred, leading to the formation of new roots. This effect was caused by the cytokinin group of substances, which stimulated cell division, resulting in the development of new shoots (28). A concentration of BA at 3 mg/L effectively induced increased shoot formation. However, a high number of shoots can lead to a decrease in the number of leaves and the length of the petiole (Fig. 2). Many plant species, including lotus, have demonstrated an increase in shoot numbers when treated with BA at a concentration of 3 mg/L. The extremely high concentrations of BA can lead to growth inhibition and result in changes in the morphological characteristics of the plant (5, 8, 29, 30).

#### **Gamma radiation sensitivity test of pink and white lotus seedlings in vitro**

A suitable gamma radiation dose exposure of pink and white lotus seedlings was determined using Probit analysis to calculate the probability of radiation dose that induced a 50% lethality rate of the total tissue ( $LD_{50}$ ). Results showed that within the first week, the non-irradiated pink and white lotus seedlings exhibited continued growth without any microbial contamination, similar to the irradiated pink and white lotus seedlings at all radiation dose levels. No tissue contamination was observed during the first week. However, after 3 weeks of cultivation, tissue mortality occurred along with bacterial contamination. By the fourth week, tissue mortality rate of both pink and white lotus seedlings did not additionally increase and microbial contamination was not further detected.

After gamma radiation exposure at rates of 0, 20, 30 and 40 Gy, the survival rates of pink lotus seedlings were



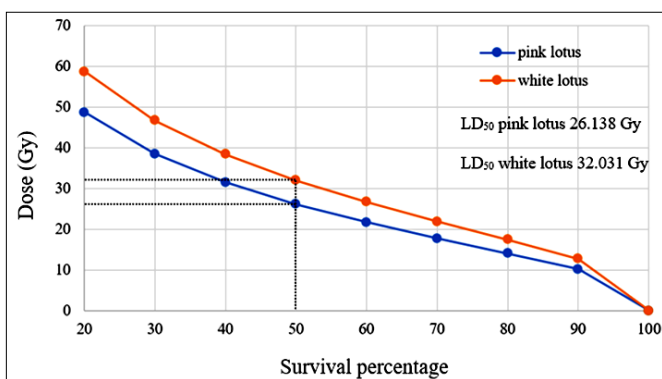
**Fig. 2.** Growth of pink and white lotus seedlings cultured in liquid MS medium supplemented with 3 mg/L BA. (A) Seedlings after one week of culture. (B) Seedlings after 4 weeks of culture. (C) Plant mortality.



100%, 67.5%, 36%, 32% and the survival rates of white lotus seedlings were 100%, 76.66%, 50% and 40% respectively (Table 3). As the radiation dose increased, both pink and white lotus seedlings showed decreased survival rates. The LD<sub>50</sub> value for pink lotus was determined to be 26.138

**Table 3.** Survival and tissue mortality rates after gamma radiation expo-

Radiation Dose (Gy)	Survival rate after gamma radiation exposure (%)	
	Pink lotus	White lotus
0	100.00	100.00
20	67.50	76.66
30	36.00	50.00
40	32.00	40.00

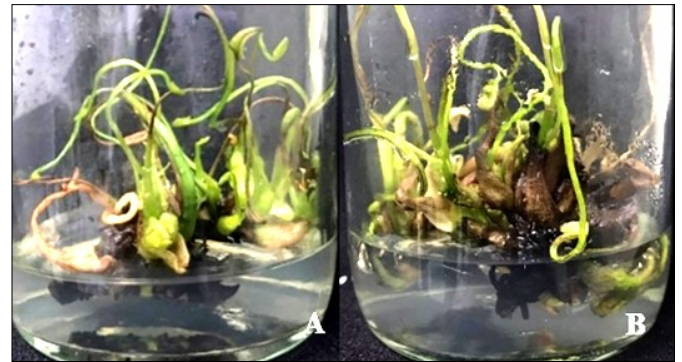


**Fig. 3.** Relationship between the radiation dosage and seedling survival percentage (LD<sub>50</sub>).

Gy, while for white lotus, it was 32.031 Gy (Fig. 3).

Gamma radiation has penetrating effects on all types of matter, causing significant damage when the energy for destruction is high (8, 31). This can result in chromosomal aberrations, leading to cell death (32) or cell arrest (33). Nonetheless, cells possess DNA repair mechanisms to reduce radiation-induced risks or other mutagenic agents, enabling them to maintain their normal functions. During the DNA repair process, some DNA segments may be lost or undergo rearrangements, which can lead to genetic mutations. If DNA damage is severe and cannot be repaired, the cells may undergo apoptosis. In this experiment, low radiation doses did not cause morphological changes or mutations; however, an increase in stem thickness and curling of leaves in irradiated seedlings without root formation was observed and exhibited growth ceased in plants (Fig. 4). Low doses of gamma radiation can modify the appearance of plants, while the utilization of excessively high radiation levels is detrimental to plants (34). According to one report low dose effects result in physiological, biochemical and molecular changes, most of which are advantageous (24). It also promotes germination and seedling development; low gamma levels may or may not have the same effects on seed germination and seedling growth. Gamma rays, functioning as ionizing radiation, exert their influence on plant growth and development by instigating cytological, biochemical, physiological and morphological alterations within cells and tissues,

primarily through the generation of free radicals. Conversely, at lower doses, they can exhibit stimulatory effects (35). Gamma radiation levels that are low cause just minor mutations that do not alter the genetic code. Unlike genetically modified organisms (GMOs), gamma irradiation is the most commonly employed physical mutagen and is



**Fig. 4.** Pink and white lotus seedlings after 4 weeks of gamma radiation exposure (A) Pink lotus (B) White lotus.

chosen for plant breeding (36).

Results showed that pink and white lotus had different LD<sub>50</sub> values, consistent with a study, who tested gamma radiation on yellow lotus bulbs and determined the median lethal dose (LD<sub>50</sub>) as 17 Gy (6). Another study also showed that gamma radiation exposure on underground stem bulbets of 'Jonkolnee' water lily resulted in an LD<sub>50</sub> of 55.6 Gy, while *Zephyranthes* spp. had an LD<sub>50</sub> of 9.8 Gy (37). It was found that gamma radiation exposure on pink and white lotus seedlings in tissue culture resulted in LD<sub>50</sub> values of 36.99 Gy and 35.34 Gy respectively, indicating that the suitable radiation dose depends on the lotus species and the tissue segment used for the radiation (8).

After gamma radiation exposure, cultivation of lotus seedlings was carried out using potting soil as the planting medium and water in the culture vessels, with no survival rate observed when the seedlings were kept in the greenhouse. After one-week, fungal contamination was observed and the seedlings showed signs of rotting, contradicted of an earlier work, as those studies reported a 95% survival rate (5). When removing the seedlings from the culture vessels, the agar was washed off, as this serves as a food source for fungi, especially under high humidity conditions that promote rapid fungal growth (8). During the transplantation of plant seedlings, covered containers should be used to control humidity in the culture vessel and minimize any adverse effects on the newly transplanted lotus seedlings as their photosynthetic ability may not be sufficient (38, 39). It was reported that the survival rate of plants decreased with an increase in gamma radiation dose, similar to findings in various flower species like bulbs and seeds when exposed to natural radiation conditions (31). In this experiment, no survival rate of lotus seedlings from both species was observed, possibly due to their weakened ability to adapt and recover to the natural environment after gamma radiation exposure. Aligning with the findings of another study, mutants plant treated to 1 and 2 krads of gamma or X-rays showed profuse adventitious roots, extended secondary roots and strong shoot

growth in addition to healthy rhizome development (31). When exposed to 3 - 5 krads of either gamma or X-rays, however, the majority of plants exhibited aberrant characteristics such as vitrification, chlorosis, petioles distorted, including a notable suppression of lateral buds, secondary roots and rhizomes. Additionally, 4 weeks after receiving 6 krads of gamma ray treatment, all plants died.

## Conclusion

In this study, seeds of pink and white lotus were surface sterilized by burning and then cultured on solid MS medium, resulting in a 100% survival rate. When the solid MS medium was supplemented with liquid MS medium along with 3 mg/L of BA, this significantly increased the number of shoots. The findings of this research can be applied to increase the yield of pink and white lotus and also help to reduce the damage caused by plant diseases, as these cultured lotus plants are disease-free propagation. Furthermore, the practical application of irradiated pink and white lotus seedlings obtained from tissue culture can serve as a new approach to developing new lotus varieties for commercial purposes, thus increasing the value of lotus plants.

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## Authors' contributions

VP conducted the design of the study, carried out the experiments. NH, SA participated in the plant collection, assistant the experiments. MD revised the manuscript. NB revised the manuscript. CW conducted the design of the study, revised the manuscript. PW performed the statistical analysis. SP conducted the design of the study and drafted the manuscript.

## Compliance with ethical standards

**Conflict of interest:** The authors here with declare no conflict of interest.

**Ethical issues:** None.

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