

STUDIES OF PRESERVATION OF LOTUS POLLENS AND INTERSPECIFIC LOTUS BREEDING

Vichai Puripunyanich^{1,*}, Piyanuch Orpong¹, Lamai Maikaeo¹,
Woranuch La-onsri², and Roppon Picha¹

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Abstract

Lotus (*Nelumbo nucifera*) pollen viability was studied under various conditions, including 30°C room temperature, 8°C refrigerator, and -20°C freezer for storage periods of 7, 10, and 14 days, respectively. The pollens were stained with acetocarmine for pollen survival testing. Lotus pollens stored for 7 days at room temperature and 8°C refrigerator exhibited the highest viability percentages (88.00% and 89.00%, respectively). Subsequently, lotus anthers were irradiated with gamma rays at 0, 30, and 60 Gy and preserved at 8°C refrigerator for 7, 10, and 14 days. Examination of pollen viability showed that there was no statistically significant difference between the viability of pollens irradiated at 30 Gy and non-irradiated treatment after 7 days. However, over a storage period of 14 days, the survival percentage of 30-Gy irradiated pollen decreased more rapidly compared to the non-irradiated. Pollens from the mutant yellow lotus *N. lutea* were collected at Queen Sirikit Botanical Garden, Chiangmai, and stored under 8°C as the optimal condition for preserving lotus pollens and transferred to Thailand Institute of Nuclear Technology, Nakhon Nayok for interspecific crosses with white native lotus flowers (*N. nucifera* 'Boontarik'). Only four plants survived and only one could flower. These cultivars successfully adapted to the Thai central plain climate and flowered after 4 months of planting. Plant characteristics were similar to Thai native 'Boontarik' lotus but its flowers showed creamy petals. This cultivar was unofficially named *Nelumbo* 'Yellow Boontarik'.

Keywords: Gamma radiation; Interspecific crossing; Lotus; Mutant; Pollen; Preservation

¹ Nuclear Technology Research and Development Center, Thailand Institute of Nuclear Technology (Public Organization), Ongkharak, Nakhon Nayok, Thailand. Email: vichaipuri@gmail.com*; piyanuch@tint.or.th; lamai@tint.or.th; aeroppon@tint.or.th

² Queen Sirikit Botanic Garden, Botanical Garden Organization, Maerim, Chiang Mai, Thailand. Email: woranuchla@hotmail.com

* Corresponding author

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Introduction

Lotus is an aquatic plant commonly found in Thailand and other Asian countries. It has been closely connected to the traditional lifestyle and cultural practices of the Thai people. For example, it is commonly used for decoration and religious ceremonies (Akkarakulthorn *et al.*, 2014). Lotus belongs to the Nelumbonaceae family which consists of the *Nelumbo* genus only. Within *Nelumbo*, there are two species: *Nelumbo nucifera*, characterized by its pink and white flowers, and *N. lutea*, distinguished by its yellow flowers (Slocum, 2005; Chomchalow *et al.*, 2007; Puripunyanich *et al.*, 2014). The standing leaves of lotus are typically large and held above the water surface, supported by long stalks.

In Thailand, local cultivars of lotuses belong to the species *N. nucifera* (Asian lotus), which is native to tropical and subtropical Asia to Australia (Mitranont, 1985; Wasuwat, 2004). These include the magnolia lotus ('Album Plenum') and Hindu lotus, both of which produce flowers with white tepals. They also include 'Roseum Plenum' and other cultivars, as well as some wild populations. The flowers of most Asian lotuses are pink to redish pink, and no cultivars or wild populations of this species produce yellow flowers.

From previous projects, the diversity of medicinal plants and herb usage in the countryside of Thailand have provided valuable insights into plant genetic conservation, propagation management, and breeding. The insights have the potential to raise awareness among local communities about the value of medicinal plants in their local area which will foster participation in conservation efforts and promote sustainable management of natural resources (Vutijurepang, 2000; Nitipon, 2011). During the survey of native lotus populations across Thailand, challenges were encountered in collecting seeds or whole plants. These challenges may be due to the several factors, such as water levels, temperature, and rainfall, which affect lotus growth and seed production. Additionally, harvesting time, wildlife, and human activities could also affect the lotus population. Sometimes only lotus anthers could be collected, but almost all of the pollens did not survive the transportation back to the laboratory, approximately one week after the collection (Bodhipadma *et al.*, 2013). Therefore, preserving pollen viability is crucial for extending shelf life and ensuring successful research outcomes.

Moreover, to potentially enhance the genetic variation of lotus pollen, mutation breeding using gamma irradiation was considered. Gamma is low

linear-energy-transfer radiation that can result in damage to molecules in the cells by ionization. Gamma radiation, even at very low doses, has been shown to induce breakages of the DNA backbones as well as oxidative damage to the nitrogenous bases of the DNA. These damages, especially DNA double-strand breaks, can lead to mutation in biological systems.

Gamma-ray can also be used as a tool to enhance the diversity of local plant cultivars (International Atomic Energy Agency, 1977; Arunyanart and Soontronyatara, 2002; Soontornyard and Puripunyanich, 2002). It was used to induce a photoperiod-response mutation in the American lotus (*N. lutea*). The irradiated rhizomes were cultivated to maturity in a mountainous region of northern Thailand. None of the plants flowered during their first year of growth. This lack of flowering may be due to the effects of gamma irradiation, the time required for maturity, and the growth conditions. Therefore, they were further propagated via rhizomes. Finally, one mutant American yellow lotus first flowered at Queen Sirikit Botanical Garden during the second year of cultivation. When the mutant plant bloomed, its stigma and anthers of which were yellow. The petals were yellow at the base, but the color was paler yellow in the middle. Also, the base of the petals appeared narrower but more obtuse at the tip. Although the flowers were fully delevoped with stamens and stigmas, seeds could not be obtained. Unlike *Nelumbo* spp., the native lotus of Thailand which can produce flowers at any time of the year, the mutant *N. lutea* only flowered from June through August when the day length is sufficiently long. In addition, the plant temporarily ceased its growth during the cool season similar to the original *N. lutea*. 'Chandrakomen' (*N. nucifera* 'Pathum' x *N. lutea*) was the first released variety in 2008 and was registered with the International Waterlily & Water Gardening Society (IWGS) in 2011. Its first-day blooming flower had pink petals that gradually turned creamy yellow shade by the last day of blooming.

Therefore, the objectives of this study were: 1) to investigate the viability of lotus pollens stored under different temperature conditions; 30°C room temperature, 8°C refrigerator, and -20°C freezer for 7, 10, and 14 days, 2) to study the viability of gamma-irradiated lotus pollen exposed to 30 and 60 Gy at 8°C, and 3) to perform interspecific crosses between *N. nucifera* 'Boontarik' and the mutant *N. lutea* to study hybridization outcomes.

Materials and Methods

Pollen viability

1. Plant materials

Pollens were collected from Asian lotus that are located in Thailand Institute of Nuclear Technology or TINT (Public Organization), Nakhon Nayok province (Figure 1).

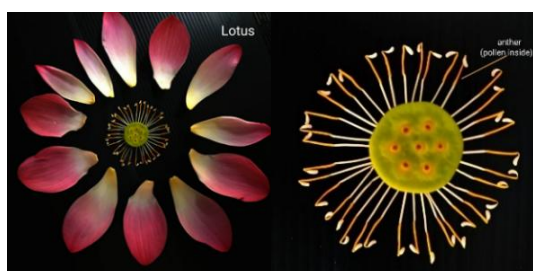


Figure 1. Lotus and its anthers

2. Viability of lotus pollens under different temperatures testing

Lotus pollens from the blooming flower stage at the second day of blooming were stored in sample bottles under three temperature conditions: room temperature at 30°C, refrigerator at 8°C, and freezer at -20°C for durations of 7, 10, and 14 days. After 7, 10, and 14 days of preservation in every treatment, pollens were gently transferred onto slides using a brush. Subsequently, pollens were stained with 1% acetocarmine, for 30 mins for viability testing (Singh, 2003). Pollen colorimetric counts were randomly examined under a 40X microscope, any red or pink-stained pollens were classified as viable or active pollen while pale or translucent pollens were classified as non-viable or inanimate pollens (Raksat, 1996). The viability percentage was calculated using the following formula:

$$\text{pollen viability (\%)} = \left(\frac{\text{number of viable pollens}}{\text{number of total pollens}} \right) \times 100$$

3. Viability testing of gamma-irradiated lotus pollens

Lotus pollens were irradiated using gamma radiation at the doses of 30 and 60 Gy from a Cobalt-60 source at TINT. The irradiated and non-irradiated pollens were kept in an 8 °C refrigerator. Then, pollens were stained with 1% acetocarmine, for 30 mins for the viability test after 7, 10, and 14 days in refrigerator preservation in every treatment. The stained pollens were randomly examined under a 40X microscope. The number of pollens was

randomly counted and compared to the total count. The red-stained pollen was classified as viable or active pollen including the pollen that was not spherical caused by irradiation influence. The pollens that were not stained red were classified as non-viable or inactive pollens. The viability percentage was calculated using the same formula as the one mentioned above. The optimum dose of gamma irradiation was calculated from the regression graph plotted between radiation dose and relative survival percentage. The relative survival percentage was calculated using the following formula:

$$\begin{aligned} \text{Relative Survival Percentage (\%)} \\ = \left(\frac{\text{Survival in treatment}}{\text{Survival in control}} \right) \times 100 \end{aligned}$$

Three repetitions were randomized in each treatment. All data were collected to calculate the survival percentage. The collected data were analyzed for variance (ANOVA) by completely randomized design (CRD) with a P-value less than 0.05 significance level and the mean of difference was compared by the Least Significant Difference (LSD) method.

Interspecific crossing over *N. nucifera* 'Boontarik' x *N. lutea*

After determining the suitable temperature for pollen collection in experiment 1, interspecific hand pollinations were conducted between *N. nucifera* 'Boontarik' and the mutant American yellow lotus (*N. lutea*) which can flower in Thailand (Figure 2). Their active pollens were obtained from three-day-old flowers after their first bloom and were collected into a plastic box with ice at Queen Sirikit Botanical Garden, Maerim, Chiangmai, Thailand. After being transported for one day from Chiang Mai to Nakhon Nayok, pollens of *N. lutea* were brushed onto the receptive stigma of *N. nucifera* 'Boontarik' at TINT. Fruits (tori) were allowed to develop to maturity in 4-5 weeks. Seeds were collected after the fruits turned brown and planted in nursery pots.



Figure 2. American yellow lotus (left) and Thai lotus 'Boontarik' (right)

Results and Discussion

Pollen viability

1. Study on the viability of lotus pollens under different temperatures

The viability of lotus pollen preserved at different temperatures (30°C room temperature, 8°C refrigerator, and -20°C freezer) and for different storage periods (7, 10, and, 14 days) exhibited similar trends. When lotus pollen cells were stained with acetocarmine, dead cells were not stained. Living cells could still appear red (Figure 3). Cells with a light red colour are cells weakened by long-term storage but have not died. After staining the lotus pollen from each treatment with acetocarmine to assess pollen survival, the results showed that lotus pollen stored for 7 days at room temperature (approximately 30°C) and in an 8°C refrigerator had the highest and statistically significant preservation percentage (88.00 and 89.00%, respectively). In contrast, pollen stored at -20°C revealed a significantly lower survival percentage of 43.47% compared to storage at 30°C and -8°C on the same day. However, after 10 days of storage, lotus pollen stored at room temperature became infected with fungi. Lotus pollen stored at 30°C room temperature tended to become contaminated with fungi after 10-14 days. Pollen stored at 8°C had a survival percentage of 55.78% after 10 days and decreased to 36.30% after 14 days of storage. Pollen stored at

-20°C had a survival percentage of 43.47% at 7 days and decreased to 22.78% and 13.33% after 10 and 14 days of storage, respectively (Table 1). Therefore, both hot and humid conditions and sub-zero temperatures are unsuitable for pollen storage.

2. Study on the viability of 0, 30, and 60 Gy gamma irradiated lotus pollens under 8°C conditions

The anthers of lotus were irradiated with gamma radiation at 0, 30, and 60 Gy and stored using the optimal preservation method identified in the first experiment, which involved storage in an 8°C refrigerator for durations of 7, 10, and 14 days. The pollen viability examination showed that at 30 Gy, lotus pollen viability was nearly equivalent to non-irradiated treatment after 7 days (90.00% and 89.40%, respectively). However, the survival percentage of pollens irradiated at 30 Gy rapidly decreased to 25.48% compared to 50.00% for the non-irradiated pollens after 10 days of storage and further decreased to 8.47% compared to 33.15% for the non-irradiated pollens after 14 days. In contrast, the 60 Gy irradiated lotus pollen exhibited a survival percentage of 33.10% after 7 days, which decreased to 5.88% after 10 days of storage and subsequently reached 0.00% (or die) after 14 days (Table 2).

Table 2. The relative survival percentage of lotus pollen in various gamma doses under 8°C preservation

Gamma dose (Gy)	Relative survival percentage (%)		
	7 days	10 days	14 days
0	90.00 ^a	50.00 ^b	33.15 ^c
30	89.40 ^a	25.48 ^c	8.47 ^d
60	33.10 ^c	5.88 ^d	0.00 ^e
F-test ^{1/}	*	*	*

^{1/} (p<0.05), * significant

Table 1. The relative survival percentage of lotus pollens in various temperature preservation

Temperature (°C)	Relative survival percentage (%)		
	7 days	10 days	14 days
30	88.00 ^a	Fungal infected	Fungal infected
8	89.00 ^a	55.78 ^b	36.30 ^c
-20	43.47 ^b	22.78 ^d	13.33 ^e
F-test ^{1/}	*	*	*

^{1/} (p<0.05), * significant

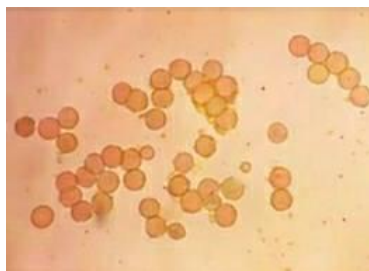


Figure 3. Lotus pollen viability observation (stained with 1% acetocarmine) under a 40X microscope showing active and inactive pollens

The survival percentage was calculated at the median lethal dose (LD₅₀) at 7 days after irradiation and preserved at 8°C condition. The resulting regression equation was determined as $y = -1.0537x + 110.31$ ($R^2 = 0.7579$). Therefore, the median lethal dose at 7 days for lotus pollens was determined to be around 57 Gy for gamma irradiation. When considering the irradiated lotus pollen cells, the three cell characteristics were observed (Figure 4): 1) The lotus pollen cells remained normal spherical. This part of the cell is considered the healthy part of the cell. There may be changes to the genes within the chromosomes, but the cell can repair all or most of the cell abnormalities. 2) Broken cells, which are dead and completely non-functional. 3) Lotus pollen cells are no longer spherical (non-spherical cells). These cells are not dead but they may show significant genetic changes within the

chromosomes, and the cells cannot repair all the abnormalities, leading to the expression of mutant characteristics.

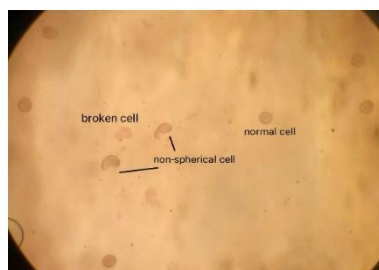


Figure 4. 30-Gy irradiated lotus pollens preserved at 8°C for 7 days

Interspecific crossing over *N. nucifera* 'Boontarik' x *N. lutea*

From the interspecific hand pollinations were conducted between *N. nucifera* 'Boontarik' and *N. lutea*, a total of 59 seeds were collected from 75 ovaries within 5 flowers. However, only five of these seeds grew into mature plants. Among the survivors, four plants (*N. nucifera* 'Boontharik' x *N. lutea*) survived and only one interspecific lotus was able to flower at TINT after four months of growth. These hybrids, resulting from the interspecific cross, exhibited similar flower shapes

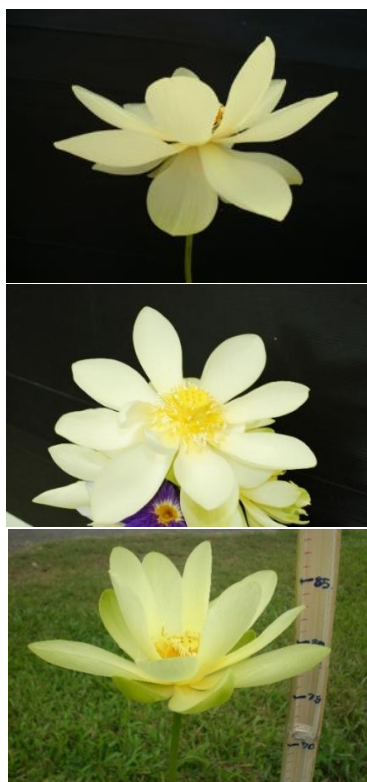


Figure 5. Yellow lotus flower blooming (*N. nucifera* x *N. lutea*)

and colors, displaying a creamy-yellow shade (yellow 2D*) lighter than their *N. lutea* parent. Lacking petaloids, they resembled single-petal lotuses. These hybrids were named 'Yellow Boontarik' (Figure 5).

Description of 'Yellow Boontarik' lotus:

- 1) Plant height: 0.70-1 meter above water level.
- 2) Blooming flower diameter: 15-20 cm.
- 3) Petals: yellow (yellow 2D*), but lighter than the American yellow lotus mutant.
- 4) Petaloids: absent.
- 5) Dormancy: no dormant period, but flowering is very few during winter (November - February).
- 6) Sterility: nearly 100%.

*The Royal Horticulture Color Chart, England.

The viability of lotus pollen was assessed after storage at different temperatures: 30°C room temperature, 8°C refrigerator, and -20°C freezer for durations of 7, 10, and 14 days. Acetocarmine staining was utilized to determine the percentage survival of pollen with living pollen staining red and inactive pollen remaining colorless. This staining method enabled the clear differentiation between viable pollen grains which show shades from pink to deep red and non-viable pollen grains which either do not stain or show very faint pink staining (Camayo-Mosquera *et al.*, 2021). Staining tests with nuclear dyes such as acetocarmine are highly accurate for determining pollen fertility which reveals the integrity of the nucleus by dyeing the chromosomes (Sunilkumar *et al.*, 2013). Raksat (1996) reported that pollen exhibiting a very dark red color indicates high enzymatic activity and readiness for germination. Lotus pollen stored at 8°C refrigerator had the highest survival percentage. However, there was a gradual decline in the number of active pollen every day. This observation suggests that lotus pollen preservation has two different effects depending on the temperature and storage period. This aligns with the findings of Satake and Yoshida (1978), who stated that low temperature affects many processes such as pollen formation, pollination, and pollen viability. Since there are no reports on the influence of temperature on the viability of lotus cultivar pollen during storage, the present report aims to provide information on the optimum storage conditions for pollen, to enhance the fertilization potential of selected lotus 'Boontarik' cultivars. These results showed that viability and pollen germination varied significantly depending on storage conditions. Variations in pollen longevity among plant species have been attributed to the difference in desiccation

tolerance of the pollen. the longevity pollen of lotus retains viability for a long time with low-temperature storage, but pollen viability generally should not be extended at the freezing temperature testing (-20°C) which indicates harmful physical and chemical changes. Therefore, lotus pollens should proceed gradually in 8°C refrigerator-stored dry pollen. The low temperature helped to slow down the changes in the intracellular activity of the pollen which is crucial for pollen viability. However, storing pollen at -20°C in deep freeze conditions may lead to the formation of icy fluid inside the cytoplasm, potentially compromising pollen viability. Therefore, -20°C storage is not considered the appropriate preservation method for lotus pollen. Interim of storage duration, the room temperature storage (30°C) was suitable for a short time lotus pollen storage and should not be stored for more than 7 days because it's very risky for fungal infection. However, the 8°C refrigerator storage could avoid fungal infection but the viability percentage also decreased every day. Therefore, although lotus pollens were stored in 8°C conditions, all lotus pollen should be used for fertilization experiment in 10 days. From the result, the viability percentage at 14 days was very low. That means we should not store lotus pollen until 14 days of preservation. Lotus pollens were stored at 8°C with approximately one-third of pollen viability only and may be infected by fungal if lotus pollens were stored at 30°C room temperature.

Mutation breeding in ornamental plants is one effective method to create many new varieties. Radiation affects DNA in several ways such as inversion, deletion, duplication, or translocation which change some genes inside chromosomes (International Atomic Energy Agency, 2018). Lotus is one of the ornamental plants for which irradiation techniques were a rapid way to induce much more plant diversity. Pollen irradiation technique paralleled with new modern technology was generally worldwide used to invent new commercial ornamental varieties. (Shin and Kim, 2007, Sengupta *et al*, 2013). In this experiment, lotus pollen was exposed to gamma radiation at doses of 0, 30, and 60 Gy and subsequently preserved using the optimal method from the first experiment at 8°C refrigerator for 7, 10, and 14 days. The preservation of lotus pollen exhibited varying effects depending on gamma radiation dose and storage duration, as suggested by the IAEA manual. The results indicated that after 7 days of preservation, lotus pollen exhibited survival percentages of 89.40% and 33.10% from 30 and 60 Gy irradiation respectively. Therefore, LD50 (the radiation dose causing 50% survival and 50% mortality) was estimated to be

approximately 57 Gy. This finding suggests that lotus pollen irradiated at a dose not exceeding 60 Gy and preserved for up to 7 days could be utilized effectively as a pollen donor in mutation breeding programs. A typical threshold for viability is at least 10-20% of the pollen grains germinating. If less than this percentage germinate, the pollen is often considered non-viable. Therefore, The pollen which was irradiated at 60 Gy of gamma radiation and preserved for more than 7 days exhibited a lower survival percentage which is not suitable for fertilization. Based on the result of this experiment, the mutant yellow lotus was cultivated at Queen Sirikit Botanical Garden in Chiang Mai, located in the northern mountainous region of Thailand. Anthers from the yellow lotus were collected and stored in an 8°C plastic box containing ice before being transported to TINT in Nakhon Nayok for crossing with a 'Boontarik' white lotus.

Conclusions

The viability of lotus pollens was assessed after preservation at 30°C room temperature, 8°C refrigerator, and -20°C freezer for durations of 7, 10, and 14 days. Lotus pollens preserved for 7 days of storage at an 8°C refrigerator and 30°C showed the highest viability; however, pollens stored at 30°C were more susceptible to fungal infection. Therefore, based on our study, the recommended storage conditions for lotus pollen should be stored in 8°C for no longer than 14 days to maintain the viability. The recommended gamma dose for lotus pollen irradiation was 30-60 Gy, provided it is stored at 8°C for no longer than 7-14 days without fungal infection. Additionally, the interspecific lotus plant (*N. nucifera* 'Boontarik' x *N. lutea*) was successfully cultivated and flowered at TINT, Nakhon Nayok. The plant exhibited similar characteristics to the Thai 'Boontarik' white lotus, including plant height, flower size, and rhizome size but their flowers showed creamy shade petals. This new cultivar has been informally named 'Yellow Boontarik'.

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