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Effects of acute gamma irradiation on the morphology of *Curcuma alismatifolia* 'Siam Shadow' and *C. alismatifolia* 'Siam Scarlet'

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Abstract

Curcuma alismatifolia (Pathumma in Thailand), also known as Siam Tulip, is an economically valuable cut flower in Thailand and is continuously bred to enhance its captivating array of colors. This study aimed to investigate the effects of acute gamma irradiation on 2 cultivars of *C. alismatifolia* 'Siam Shadow' and *C. alismatifolia* 'Siam Scarlet' for *in vitro* propagation and determination of the lethal dose 50 (LD_{50}). The irradiation was conducted at doses of 0, 20, 30, 40 and 80 Gy using young shoots tissue culture. The results of the experiment revealed that the LD_{50} for 'Siam Shadow' could not be determined while 'Siam Scarlet' was 29.06 Gy. 'Siam Shadow' exhibited chimera leaf formation when exposed to a gamma irradiated dose of 40 Gy, while 'Siam Scarlet' exhibited chimera leaf formation when gamma irradiated at doses of 20 Gy *in vitro*. After planting in a greenhouse, 'Siam Shadow' was irradiated with 30 Gy of gamma irradiated, had reduced bract numbers and the bracts became smaller. Conversely, 'Siam Scarlet' irradiated with 20 Gy of gamma irradiated, produced smaller inflorescences with only 2 bracts, while irradiation with 30 Gy resulted in 2 lobes and spotted leaves at the edges of the leaves. The results indicated that both the *C. alismatifolia* cultivars and gamma irradiation rates had a significant influence on survival rate, number of shoots, plant height, chlorophyll mutants and morphology. As the outcome, it was determined that gamma irradiation with optimum doses of gamma rays could induce new characteristics in *C. alismatifolia*.

Keywords

Acute gamma rays; *Curcuma alismatifolia* 'Siam Shadow'; *C. alismatifolia* 'Siam Scarlet'; LD_{50}; Mutation

Introduction

Curcuma alismatifolia Gagnep. is a genus of *Curcuma* belonging to the family Zingiberaceae with a rhizome as an underground stem. This plant thrives and blooms particularly well during the rainy season. Internationally, *C. alismatifolia* is often referred to as Siam Tulip because of its beautiful flowers similar to European tulips. Thailand exports Pathumma (*C. alismatifolia*) with a value range from 3-6 hundred thousand dollars in various forms, including undeveloped rhizomes, growing rhizomes and fresh flowers (1). Because *C. alismatifolia* has unique flowers and a variety of colors, it is used for cut flowers, potted plants and ornamental flowering plants, making *C. alismatifolia* interesting for many foreign countries. Thailand is an important source of the genetics and diversity of *C. alismatifolia* plants and it is found in all regions throughout the country (1, 2).

C. alismatifolia is Thailand's second-most important economic flowering plant after Orchids. It is an economic plant contained in the National Economic and Social Development Plan for exports (3). According to the producers and developers of KP Holland, *C. alismatifolia* has been exported to the Netherlands market and is worth more than 4 million euros (4). According to the report to date, the trend of production for flowering and ornamental plants in the global market is expected to include products such as flowering plants and exotic local plant species (typical local flowers and plants). Consequently, there is continuous breeding of ornamental plants to meet market demands. As a result, the breeding of ornamental plants is taking place continuously to respond to the demands of the market. The breeding of *C. alismatifolia* takes a long time because *C. alismatifolia* has a dormancy of 2-3 years. The resulting hybrids are often sterile, which is a natural mechanism and they cannot be used for further breeding. As a results, crossbreeding has not been successful (5) and the main obstacle to producing *C. alismatifolia* rhizomes at the export level is the shortage of disease-free *C. alismatifolia* rhizomes. *C. alismatifolia* rhizomes stored for planting in the next season may be inferior in quality or have infectious diseases, which will adversely affect farmers' plots and influence exports (6). Artificial crossbreeding has produced many *Curcuma* variants with superior growth properties and bract color. Inter-specific hybridization faces a significant challenge because of their low fertility, which prevents them from serving as parents for the following stages of reproduction (7). It was reported that the hybrids with near parent relationships had much higher fruit setting rates than those with distant parent relationships (8).

The impact of gamma irradiation on the germination and growth attributes of economically important plants demonstrates the potential of this technique to expedite the breeding process and facilitate the cultivation of plant varieties possessing desired characteristics (9). There were *C. alismatifolia* breeding and improvement by gamma irradiation to find a lethal dose of 50 (LD_{50}). Studies are on the result of gamma irradiation on *C. alismatifolia* hybrid 'Laddawan' in the tissue culture and found that the optimal gamma irradiating doses ranged from 30-60 Gy (1). For *C. alismatifolia* cultivars 'Pink' an irradiation dose of approximately 25 Gy was applied (10); LD_{50} values of the cultivars were achieved at 21 Gy for 'Chiang Mai Red', 23 Gy for 'Sweet Pink', 25 Gy for 'Kimono Pink', and 28 Gy for 'Doi Tung 554' (11).

Propagation through plant tissue culture techniques enables the rapid generation of large quantities of genetic material within a short period. This method ensures disease-free propagation, reduces planting duration and eliminates the dependency on seasonal factors (12). Therefore, this research examined *C. alismatifolia* breeding by using tissue culture techniques from young shoots and the seedlings were irradiated with gamma-rays, which is the process of inducing genetic mutations in plants and finding the appropriate amount of gamma irradiation to induce new characteristics. The production of varieties is produced *in vitro* (clean culture) so it is convenient for storage and can be produced out of season, which will increase income for farmers who grow *C. alismatifolia* in the future.

Materials and Methods

Plant materials

Plant samples used in this experiment were 2 cultivars of *C. alismatifolia* 'Siam Shadow' and 'Siam Scarlet' were obtained from *Curcuma* Farmers in San Sai district, Chiang Mai, Thailand (13, 14). These samples were cultured *in vitro* at the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand.

Rhizome sterilization

The buds from the rhizomes of *C. alismatifolia* (adapted from the patent code IPCA01H4/00, C07K) (6) were cut and then disinfected with 70 % ethanol (v/v) for 1 min before discarding the solution. Next, bleach disinfection was performed using a solution containing 20 % (v/v) sodium hypochlorite along with 2 drops of Tween 20, which was shaken at 150 rpm for 20 min and then discarded. The explant was placed on MS medium (15), 30 g sucrose, 2.75 g Kelcogel®, adjusted pH to 5.6 supplemented with Thidiazuron (TDZ) at a concentration of 1 mg/L. The explant was cultured at 25 ± 2 °C with a light intensity of 65 $\mu mol/m^{-2}/s^{-1}$ for 16 h/day using fluorescent white light.

After culturing for 4 weeks, the young shoots free from microbial contamination were selected and cut in half lengthwise. These shoots were cultured on MS medium with plant growth regulators BA at a concentration of 3 mg/L for an additional 4 weeks at 25 ± 2 °C with a light intensity of 65 $\mu mol/m^{-2}/s^{-1}$ for 16 h/day using fluorescent white light.

LD_{50}: The impact of gamma irradiation on tissue death

Seedlings of 'Siam Shadow' and 'Siam Scarlet' were irradiated with gamma rays at rates of 0, 20, 30, 40 and 80 Gy. The gamma irradiation source was cobalt-60 and consisted of 6 rods provided by Paul Stephens Consultancy Ltd. (at a dose rate of 75 Gy/h, JS 8900 IR-155 at the Thailand Institute of Nuclear Technology (Public Organization) in Ongkharak District, Nakhon Nayok Province, Thailand. Subsequently, the seedlings were placed on MS medium supplemented with BA 3 mg/L cultured at 25 ± 2 ºC under 50 % light intensity for 1 week. After irradiation, the number of surviving plantlets was recorded to calculate the LD_{50}, after culturing for 4 weeks. The results of morphological changes were recorded.

Survival rate and mutation after transplanting seedling

The temperature in the tissue culture bottles was adjusted to room temperature for a day. The seedling was placed in a container with planting materials including coconut coir, husk, black husk ashes and potting soil at a ratio of 3:4:2:1. The planting materials were filled to ¾ of its volume to cultivate the seedling in the greenhouse.

Statistical analysis

Statistical analysis was performed using SPSS version 24.0, with a confidence level of 95 %. The experimental design was a factor in a completely randomized design (Factorial in CRD) with 2 factors: factor A: cultivars of *C. alismatifolia* ('Siam Shadow' and 'Siam Scarlet'), factor B: gamma irradiation dose on the number of shoots after irritation, height of pseudo-stems and survival rate after planting with 3 replications for each treatment, 25 plantlets per replication. The paired difference mean was then compared by Scheffe's test and determined LD_{50} by Probit analysis.

Results and Discussion

Effects of gamma irradiation on the LD_{50} and morphology *in vitro*

The buds from the rhizomes of 'Siam Shadow' and 'Siam Scarlet', which were disinfected had survival rates of 92.86 % and 85.72 % respectively, with one shoot/explant and a plant height of 2-3 cm. The explant, which had undergone gamma irradiation for 4 weeks, showed normal growth. Notably, gamma irradiated 'Siam Shadow' seedlings had a higher survival rate than 'Siam Scarlet' seedlings under the same dose of gamma irradiation, with no microbial contamination detected (Fig. 1).

Table 1. Survival rate and number of shoots of 'Siam Shadow' and 'Siam Scarlet' seedlings after gamma irradiation *in vitro*.

Dose rate (Gy)	Survival rate (%)		No. of shoots/explant	
	Siam Shadow	Siam Scarlet	Siam Shadow	Siam Scarlet
Control	100 ± 0.00^{d}	100 ± 0.00^{d}	2.52 ± 0.15^{e}	1.48 ± 0.19^{d}
20	100 ± 0.00^{d}	88 ± 4.90^{c}	2.08 ± 0.10^{e}	1.20 ± 0.06^{cd}
30	100 ± 0.00^{d}	64 ± 7.48^{b}	1.44 ± 0.32^{d}	0.88 ± 0.10^{b}
40	100 ± 0.00^{d}	0 ± 0.00^{a}	1.12 ± 0.10^{bc}	0.00 ± 0.00^{a}
80	64 ± 4.00^{b}	0 ± 0.00^{a}	0.95 ± 0.30^{b}	0.00 ± 0.00^{a}
cultivars (A)	*		*	
dose (B)	*		*	
A x B	*		*	

* There was a statistically significant difference at the 95 % confidence level. The mean ± standard deviation followed by different superscripts within the same column was statistically different by Scheffe's test.

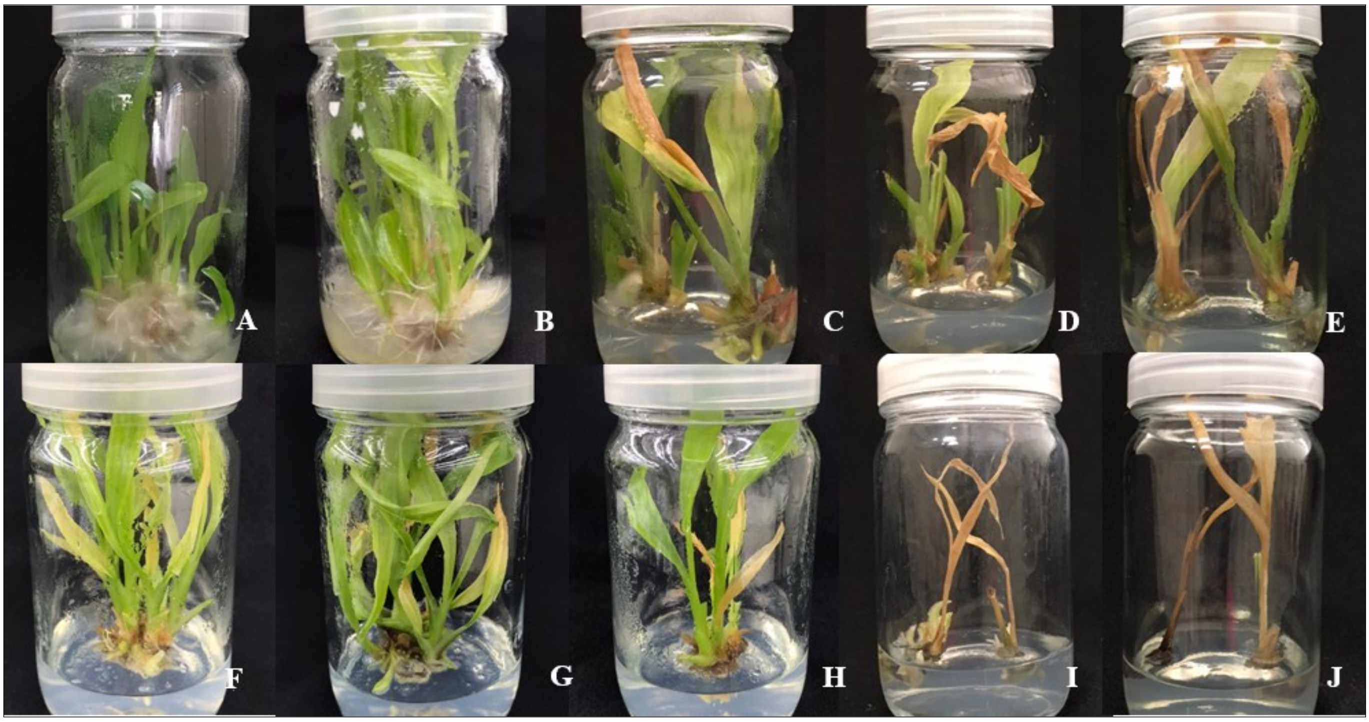

Fig. 1. Growth of 'Siam Shadow' and 'Siam Scarlet' seedlings after gamma irradiation on MS medium supplemented with BA 3 mg/L for 4 weeks. (**A-E**) Seedling of 'Siam Shadow': Control, 20, 30, 40 and 80 Gy and (**F-J**) Seedling of 'Siam Scarlet': Control, 20, 30, 40 and 80 Gy.

Seedling of 'Siam Shadow', the control and irradiated by gamma rays at doses of 20, 30, 40 and 80 Gy exhibited survival rates of 100 %, 100 %, 100 %, 100 % and 64 % respectively. Because the survival values were greater than 50 %, LD_{50} could not be determined. On the other hand, seedlings of 'Siam Scarlet', including the control and irradiated by gamma rays at doses of 20, 30, 40 and 80 Gy, displayed survival rates of 100 %, 88 %, 64 %, 0 % and 0 % respectively (Table 1). With Probit analysis, LD_{50} was determined to be 29.06 Gy. The seedling of 'Siam Shadow' had a higher survival rate than the seedling of 'Siam Scarlet' under the same irradiation level because of radiosensitivity differences. As a result, the amount of gamma irradiation applied to each plant is not the same. The same species, but different varieties, exhibit sensitivity to different irradiation doses (16, 17). It was reported that low-dose impacts caused physiological, biochemical and molecular changes, the majority of which were positive (18). Low gamma-ray levels may or may not have the same effects on seedling growth, but they also increase germination and seedling growth. Gamma-rays, which act as ionizing radiation, can cause cytological, biochemical, physiological and morphological changes within cells and tissues, principally through the production of free radicals. These changes have an impact on how plants grow and develop. On the other hand, they can have stimulatory effects at lower doses (19).

The chances of survival by *C. alismatifolia* are lower as the amount of gamma irradiation increases because gamma irradiation has a penetrating effect on the object. As the dose increases, the destructive energy is high, resulting in the death of plant tissues such as *Lindernia* spp. (20) *Narcissus tazetta* (21) *Cullen corylifolium* (22) *Curcuma* hybrid 'Laddawan' (23). When plants are exposed to gamma irradiation, it results in chromosomal abnormalities and may lead to a reduction or a halt of cell division (2). High doses of gamma irradiation can completely inhibit the growth of the apical meristem, causing a cessation of cell expansion (24). Growth reductions can result from auxin degradation, auxin production inhibition, assimilatory mechanism failure and changes in the particular activity of enzymes.

The number of shoots for 'Siam Shadow' and 'Siam Scarlet' seedlings on MS medium supplemented with BA 3 mg/L indicated that the control of 'Siam Shadow' and 'Siam Scarlet' had average amounts of 2.52 ± 0.15 and 1.48 ± 0.19 shoots/explant respectively. The seedling of 'Siam Scarlet' at a dose of 40 Gy gamma irradiation had the minimum number of shoots at 0.88 ± 0.10 shoots/explant, while the seedling of 'Siam Scarlet' at a dose of 40 and 80 Gy gamma irradiation had no shoot because the seedling died (Table 1). Following the research conducted the irradiation was made on *C.* hybrid 'Laddawan' seedlings with gamma rays, it was found that the number of shoots decreased with an increase in gamma irradiation dose (1). This finding contrasts with another report (25), who reported that the irradiation doses of 20 and 40 Gy could stimulate tillering of *Globba williamsiana* more than the control and decreased as the dose of irradiation increased. Appropriate doses of irradiation reduce the level of auxin hormone, inhibit the growth of the crest, and promote the growth of the lateral bud (26). The quantities of gamma radiation administered and the specific plant types employed greatly influence both the number of viable plants and several aspects of plant growth (27). According to one report, increases in root and shoot lengths were seen in plants after seeds were exposed to low levels of gamma radiation (28). This growth stimulation is caused by the transcriptional regulation of genes associated with phytohormones, the antioxidant system, late embryogenesis abundant proteins, and cell wall components. However, cultivars of *C. alismatiforia* ('Siam Shadow' and 'Siam Scarlet') and the dose of gamma irradiation have a mutual influence on the renewal of *C. alismatiforia* with a statistically significant difference at the 95 % confidence level.

In vitro, notable changes in leaf appearance were observed in 'Siam Shadow' at an irradiated dose of 30 Gy. The leaves were light green in color, which indicates an unstable appearance (Fig. 2). This characteristic was not found in the new shoots that emerged. At an irradiated dose of 80 Gy, 'Siam Shadow' had a pale-white characteristics and the white plant was eventually found dead. Also, the characteristics of chimera leaves were observed in the 'Siam Shadow' irradiated by 40 Gy of gamma-ray and 'Siam Scarlet' irradiated by 20 Gy (Fig. 2) which was similar to *Curcuma* hybrid (23) *C.* hybrid 'Laddawan' (1). The irradiated plants exhibited lower chlorophyll content compared to the control, with a further decrease in chlorophyll content as the irradiation dose increased. These chimeric leaves displayed irregular changes. It is important to note that most somatic mutations are not inheritable, meaning that the next generation will no longer exhibit these variations (10). It was reported that gamma-rays interact with atoms or molecules to create free radicals in living cells (29). Depending on the intensity of irradiation, these radicals have been shown to differentially influence the morphology, anatomy, biochemistry and physiology of plants. They can harm or modify critical plant cell components. Among these consequences are modifications to the metabolism and cellular structure of plants.

Effects of gamma irradiation on survival rate and morphology in vivo

Before planting the seedlings of 'Siam Shadow' and 'Siam Scarlet' after gamma irradiation in the greenhouse, they were acclimated to room temperature for one day. After that, agar gel of the tissue culture medium was removed and the seedlings were soaked in an antifungal solution for 1 min. Following this, the seedlings were placed in a container with planting materials including coconut coir, husk, black husk ashes and potting soil at a ratio of 3:4:2:1.

After planting in a greenhouse for 4 weeks, the survival rates of 'Siam Shadow' and 'Siam Scarlet' seedlings were observed except for seedlings of 'Siam Scarlet' that died after gamma irradiation at 40 and 80 Gy. The survival rate of

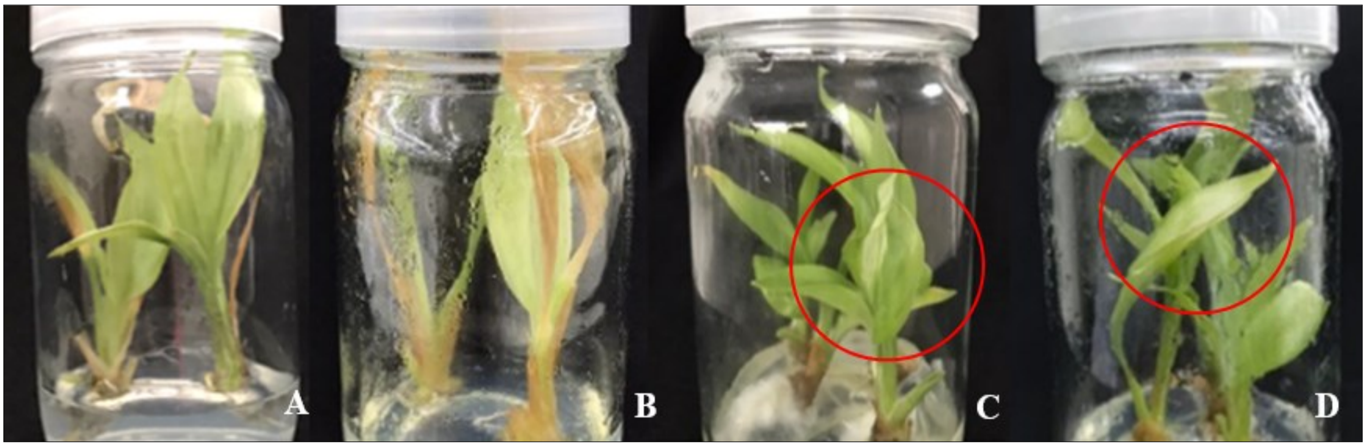

Fig. 2. Seedlings of 'Siam Shadow' and 'Siam Scarlet' after gamma irradiation on MS medium supplemented with BA 3 mg/L for 4 weeks. (**A-B**) Leaf characteristics of 'Siam Shadow' at doses of 30 and 80 Gy, (**C**) Leaf characteristics of 'Siam Shadow' at doses of 40 Gy and (**D**) Leaf characteristics of 'Siam Scarlet' at dose of 20 Gy.

seedlings of 'Siam Shadow' on control, 20, 30 and 40 Gy were found at 100 %, 100 %, 88 % and 84 % respectively. No survival of seedling of 'Siam Shadow' when it was irradiated at 80 Gy. On the other hand, the survival rate of seedlings of 'Siam Scarlet' in control, 20 Gy and 30 Gy was found at 100 %, 100 % and 62.50 % respectively. These findings align with a study on *Catharanthus roseus* cv. Mediterranean Deep Rose, which was subjected to acute gamma irradiation at a quantitative 50-200 Gy (30). It was found that the survival rate decreased as the irradiation dose increased.

The heights of 'Siam Shadow' and 'Siam Scarlet' seedlings were observed after 4 weeks of planting in the greenhouses. The control 'Siam Shadow' and 'Siam Scarlet' seedlings had the average maximum pseudo-stem heights of 21.91 ± 0.61 and 18.96 ± 0.88 cm respectively (Table 2 and Fig. 3). The height of the stem decreased as the irradiation dose increased corresponding to one reported that the height of the *Gomphrena* Hybrid plant after irradiation had statistically and significantly decreased (31). *Curcuma* Hybrid height decreased as the gamma irradiation dose increased because of the response of plant tissue to gamma irradiation (23). Gamma irradiation, especially at lower doses, has gained widespread recognition for its ability to enhance the growth attributes of numerous economically important plants (32). It has been statistically analyzed that the cultivars of *C. alismatifolia* ('Siam Shadow' and 'Siam Scarlet') and gamma irradiation have mutual influence and affect the survival rate and height of pseudo-stem with a statistical significance at a 95 % confidence level.

Table 2. Survival rate and height of 'Siam Shadow' and 'Siam Scarlet' seedlings after gamma irradiation *in vivo*.

Dose rate (Gy)	Survival rate (%)		No. of shoots/explant	
	Siam Shadow	Siam Scarlet	Siam Shadow	Siam Scarlet
Control	100 ± 0.00^{d}	100 ± 0.00^{d}	21.91 ± 0.61^{e}	18.96 ± 0.88^{d}
20	100 ± 0.00^{d}	100 ± 0.00^{d}	21.21 ± 0.18^{e}	13.81 ± 0.75^{c}
30	88 ± 1.33^{c}	62.50 ± 4.43^{b}	14.39 ± 0.71^{c}	8.02 ± 1.25^{b}
40	84 ± 2.69^{c}	0 ± 0.00^{a}	10.34 ± 0.52^{b}	0.00 ± 0.00^{a}
80	0 ± 0.00^{a}	0 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
cultivars (A)	*		*	
dose (B)	*		*	
A x B	*		*	

* There was a statistically significant difference at the 95 % confidence level. The mean ± standard deviation followed by different superscripts within the same column was statistically different by Scheffe's test.

After planting in greenhouses, changes in the flower morphology of 'Siam Shadow' were observed at 30 Gy of gamma irradiation. The bracts and flowers separated from the inflorescences and the number of bracts decreased. 'Siam Shadow' at the 40 Gy of gamma irradiation had withered flowers. For 'Siam Scarlet' exposed to 20 Gy of gamma irradiation, small inflorescences and 2 bracts were observed (Fig. 4), consistent with one study (33). The dose of gamma irradiation of 10-30 Gy changed the morphology of the flower, the number of layers of petals and flower color. These abnormalities may manifest themselves through the phenotype after irradiation. This is caused by irradiation affecting gene changes. If one of the genes is abnormal, it can affect the formation of pigments in plants (34).

After planting the 'Siam Scarlet' that had undergone 30 Gy of gamma irradiation, there were 2-lobed leaves and a spotted leaf appearance (Fig. 4A, 4B). Such features were the result of the function to reduce the irradiation, chemi-

Fig. 3. The seedling of 'Siam Shadow' and 'Siam Scarlet' after gamma irradiation and planted in pots for a period of 4 weeks. (**A-E**) 'Siam Shadow' seedling: control, 20, 30, 40 and 80 Gy, (**F-H**) 'Siam Scarlet': control, 20 and 30 Gy.

Fig. 4. Characteristics of 'Siam Shadow' and 'Siam Scarlet' flowers after planting in greenhouses. (**A**) 2-lobed blade of 'Siam Scarlet' (30 Gy), (**B**) Spotted leaves of 'Siam Scarlet' (30 Gy), (**C**) 'Siam Shadow' control , (**D**) 'Siam Scarlet' control , (**E**, **F**, **G**) Change of 'Siam Shadow' at petals, inflorescences and bracts (30 Gy) and (**H**) Small inflorescences and 2 bracts of 'Siam Scarlet' (20 Gy).

cal, and mutation. If the cell cannot be repaired however, it will cause a mutation (35). Gamma irradiation will cause mutations or alterations in the genetic material of cells and can transmit this change to the offspring cells (36). Irradiation has proven to be successful in mutation breeding across various crops and ornamental plants (10, 37), resulting in morphological changes in the color and shape of the stem, leaves and flowers. This process has led to the generation of novel genetic variations (38, 39), with new characteristics exclusively emerging in the treated plants (40, 41).

Conclusion

Gamma irradiation doses and cultivars of *C. alismatifolia* 'Siam Shadow' and 'Siam Scarlet' mutually influence the induction of shoots, as well as the height of pseudo-stem and survival rate after planting. The seedling of 'Siam Scarlet' LD_{50} is 29.06 Gy. Changes in leaf morphology were observed and found to have light green leaves and a pale white stem for 'Siam Shadow'. Chimera leaf formation was found *in vitro* when irradiated at 40 Gy of gamma irradiation, while ornamental leaves and flowers separated from the inflorescence decreased. Wilted flowers were found when 'Siam Shadow' was irradiated at 30 Gy. On the other hand, 'Siam Scarlet' that was irradiated at 20 Gy of gamma irradiation was found in the chimera leaves *in vitro*. After planting, the inflorescence was small, with 2 ornamental leaves. At gamma irradiation of 30 Gy, there were 2-lobed leaves and spotted leaf formation. The heights of pseudo-stems were reduced, unlike the control. This study included the guidance of *C. alismatifolia* breeding by irradiation of seedlings obtained from plant tissue culture of *C. alismatifolia* 'Siam Shadow' and 'Siam Scarlet', with the aim of providing initial towards developing new characteristic for farmers.

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Authors' contributions

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