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Research article

Inducing genetic diversity of *Anubias nana* using gamma rays

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Abstract

Plantlets of *Anubias nana* cultured on Murashige and Skoog medium were irradiated with gamma rays for both acute and chronic treatments of 0 Gy, 10 Gy, 20 Gy, 30 Gy, 40 Gy, 50 Gy and 60 Gy at a dose rate of 3.73 Gy/min (acute) and of 0 Gy, 26.88 Gy, 39.39 Gy, 66.22 Gy, 84.15 Gy, 105.52 Gy and 123.45 Gy at a dose rate of 0.45 Gy/h (chronic). Both irradiation methods were applied at the Nuclear Technology Research Center, Kasetsart University, Bangkok Thailand. At 60 d after irradiation for acute irradiation, the survival percentage of *A. nana* in the M_1V_1 generation decreased when the radiation doses increased; it was not possible to calculate the 50% lethal dose at 60 d after irradiation ($LD_{50\ (60)}$) but there was a highly significant ($p < 0.01$) decrease in the growth percentage. A dose of 34 Gy produced a 50% reduction in the growth rate at 60 d after irradiation. The selected mutants in the M_1V_4 generation had variegated leaves, dwarfism, light green leaves, abnormal leaves and albinism. With chronic irradiation in the M_1V_1 generation, there was no differences between the survival and growth percentages at doses of 0–84.15 Gy but there was with the 105.52 Gy and 123.45 Gy treatments. It was not possible to calculate the $LD_{50\ (60)}$. Nevertheless, the growth percentage at the maximum dose (123.45 Gy) was 54.38%, which was nearly equal to a 50% growth reduction. In the M_1V_4 generation, some mutants were selected to resemble acute irradiation. Therefore, the appropriate doses of acute and chronic irradiation to induce mutation of *A. nana* should be in the ranges 10–35 Gy and 80–130 Gy, respectively.

Introduction

Aquatic plants are an export product in high demand every year (Komsan, 2003). Thailand has a tropical climate and a suitable environment for the growth of aquatic plants (Komsan, 2003). The volume and export value of aquatic plants in 2014 for Thailand was 9,185,066 plants worth USD 1,141,467,072 and in 2015 was 9,706,713 plants worth USD 1,179,318,765, with the primary export market being the United States followed by Russia, Japan, Poland, the United Kingdom and Germany, respectively (Department of Agriculture, Agricultural Service Export Group, 2015). In 2015, the top-5 aquatic plant genera by export value were *Elodea*, *Hygrophila*, *Anubias*, *Echinodorus* and *Selaginella*, respectively (Department of Agriculture, Agricultural Service Export Group, 2015). *Anubias nana* is an aquatic plant that is characterized by a short stem that can grow up to 15 cm in height. The leaves are thick, oval, dark green and not more than 6 cm in length (Meanakarn and Pongchawee, 2000). The plant grows well under high humidity with a temperature of about 25°C and requires a low light level (Muhlberg, 1982; Allgayer and Teton, 1987). It can be propagated by separating the shoots or dividing the rhizome and cultivating new shoots. (Rataj and Horeman, 1977; Cook, 1996), making it suitable for decoration in an aquarium. Therefore, breeding

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mutations of *A. nana* for varieties both beautiful and quaint increases the market value of this plant. The technology of tissue culture used for the *in vitro* propagation method is rapid and can produce pathogen-free plantlets in large quantities, compared to asexual propagation by other means, such as cutting, layering or isolation (Tangpong et al., 2009). Plant propagation is also applied for plant breeding, especially when plants can be propagated asexually to create new varieties that exhibit genetic diversity or have botanical characteristics that are different from the original appearance and are more beautiful with greater variety in terms of shape and colors of leaves and flowers in response to consumer demand in addition, while *in vitro* mutagenesis has advantages compared to conventional breeding methods such as a high mutation frequency, uniform mutagen treatment, application of selective agents to a homogenous cell population, use of single cell systems and a requirement of less space to handle a large population within a short time while keeping the plant material disease free (Maliga, 1984; Harten, 1998; Suprasanna et al., 2010). Currently, *in vitro* mutagenesis uses irradiation for the induction of genetic diversity. The current research made use of both acute and chronic gamma irradiation because the advantages in terms of producing new mutant varieties of both acute and chronic gamma irradiation are a higher mutation rate than from natural mutation in order to achieve desirable characteristics that are not found in nature while also having good characteristics so that the products can be used as a new species directly in a relatively short period of time (Wongpiyasatid, 2007). Additional benefits are the improvement of original varieties in terms of their plant characteristics and the flower or leaf color, as well as reduced time and cost compared to other plant breeding methods (Harten, 1998). Plant breeding by induced mutation using gamma irradiation is one breeding approach to increase the mutation rate compared to natural mutation. The current research studied the effect of gamma irradiation on the genetic diversity of *A. nana.*

Materials and Methods

Propagation of Anubias nana in tissue culture

Pathogen-free plantlets of *A. nana* were obtained from the original mother plant starting from sterile tissue culture that was separated into single plants. The top part was cut off each plantlet to create new shoots that were cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with gelatin (1.8 g/L) for 8 wk before being subjected to acute gamma irradiation and chronic gamma irradiation.

Acute and chronic gamma irradiation

For the acute gamma irradiation treatments, plantlets of *A. nana* grown *in vitro* for 8 wk were exposed to gamma radiation at 0 Gy (control), 10 Gy, 20 Gy, 30 Gy, 40 Gy, 50 Gy and 60 Gy (at a dose rate of 3.73 Gy/min) from a Cs-137 source using a MARK I Research Irradiator. The *in vitro* plantlets of *A. nana* were exposed to chronic gamma rays at a dose rate of 0.45 Gy/h and placed at a distance of 1.5 m from a Co-60 source in the Gamma Room at the Nuclear Technology Research Center (NTRC), Faculty of Science, Kasetsart University, Bangkok, Thailand. The samples received 0 Gy (control), 26.88 Gy, 39.39 Gy, 66.22 Gy, 84.15 Gy, 105.52 Gy and 123.45 Gy of radiation for the different treatment levels. After irradiation, the plantlets were transferred to new bottles with MS medium and separated into single plants; the tips were cut off to encourage new shoots. The gamma-irradiated shoots were subcultured for four generations (M_1V_1 to M_1V_4) to obtain potential mutant lines.

The experiment was set up following a completely randomized design with three replications. Survival plantlets were recorded to calculate the $LD_{50\ (60)}$ (50% lethal dose at 60 d after irradiation). The numbers of new shoots were recorded at 60 d after irradiation for the M_1V_1 generation and the types of mutations were recorded for the M_1V_4 generation.

Statistical analysis

The data were analyzed using analysis of variance after which means were compared using the least significant difference and compared at a significant level of $p < 0.05$.

Results and Discussion

Effect of acute gamma irradiation of Anubias nana in the M_1V_1 generation at 60 d after irradiation

Effect of acute gamma irradiation on survival

When the radiation dose increased, the survival percentage decreased; however, at the maximum radiation dose of 60 Gy, the plantlet survival percentage was 75.92% (Table 1) and thus, it was not possible to calculate the $LD_{50\ (60).}$ The plantlet survival percentage was significantly ($p < 0.01$) different from the control. Doses of 0–30 Gy did not result in any significant differences with regard to the survival percentage; however, when the radiation dose was higher (40–60 Gy), the plantlet survival percentage decreased (Fig. 1), which was consistent with the findings for *in vitro* culture of *Cryptocoryne wendtii* “brown” where after exposure to acute gamma irradiation at doses of 0 Gy, 10 Gy, 20 Gy, 30 Gy, 40 Gy and 50 Gy at 60 d after irradiation, it was found that as the radiation dose increased, the plantlets survival percentage decreased (Jompuk et al., 2009) because the higher radiation dose caused direct damage through the radiation energy transferred to molecules of genetic material or to biochemicals within cells, resulting in abnormal genetic material and activity within the cells. In addition, indirect damage may have resulted from the radiation reacting with the water in the cell resulting in free radicals, which can damage plant cells (Lamseejan, 1997). However, the current results were different from experiments using *in vitro* culture of *Typhonium flagelliforme* Lodd. after exposure to gamma irradiation at doses of 0 Gy, 10 Gy, 20 Gy, 30 Gy, 40 Gy and 50 Gy at 8 wk after irradiation, where the calculated $LD_{50\ (60)}$ for gamma irradiation was 25 Gy (Sianipar et al., 2013)

Table 1 Survival percentage and growth rate percentage of *Anubias nana* in the M_1V_1 generation at 60 d after acute irradiation

Radiation dose (Gy)	Total number irradiated	Number of surviving plantlets	Plantlet survival (% of control)	Number of new plantlets	Growth rate (% of control)
0 (Control)	22	22	100.00 ± 0.00[a1]	16	100.00 ± 0.00[a]
10	37	37	100.00 ± 0.00[a]	21	72.67 ± 24.15[ab]
20	31	25	93.33 ± 11.55[a]	16	85.98 ± 10.36[a]
30	38	38	100.00 ± 0.00[a]	15	51.43 ± 26.19[bc]
40	38	31	81.74 ± 2.75[b]	12	46.56 ± 9.70[bc]
50	40	30	75.28 ± 10.26[b]	11	41.62 ± 17.28[c]
60	29	22	75.92 ± 5.25[b]	10	50.79 ± 14.04[bc]
F test	-	-	**	-	**
C.V. (%)	-	-	6.99	-	26.12
$LSD_{(0.05)}$	-	-	10.95	-	29.35

C.V. = coefficient of variance; ** = significant at 1% level

[1] = data within columns, followed by a common lowercase letter are not significantly different ($p > 0.05$)

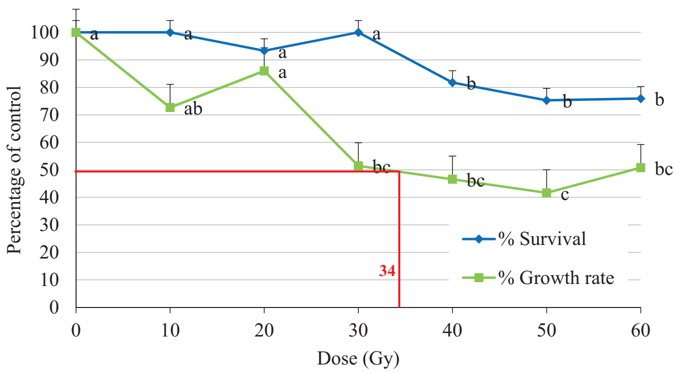

Fig. 1 Effects of radiation dose on survival percentage and growth rate percentage of *Anubias nana* plants in the M_1V_1 generation at 60 d after acute irradiation; Note LD_{50} determined from the graph at a dose of 34 Gy

Effect of acute gamma irradiation on growth

As the radiation dose increased, the plantlet growth percentage decreased by 50 percent compared with the control. The dose producing a 50% reduction in the growth rate at 60 d after irradiation ($GR_{50\ (60)}$) was 34 Gy (Fig. 1). Radiation doses of 0–20 Gy did not result in a significant difference in the percentage of growth; however, there was a significant ($p < 0.01$) difference when the *A. nana* plantlets were exposed to radiation doses of 30–60 Gy (Table 1). This was consistent with the findings of Pongchawee et al. (2007) with plantlets of *A. nana* Engler and *A. congensis* N.E. Brown after 4 wk exposure to acute gamma radiation at doses of 0 Gy, 20 Gy, 40 Gy, 60 Gy, 80 Gy, 100 Gy and 120 Gy followed by culture for 8 wk, where the growth decreased by 30-50% (34.56 Gy and 28.30 Gy, respectively) and as the radiation dose increased, the height of shoots, number of shoots and number of roots decreased. Fan et al. (2014) reported that for plantlets of *Zizania latifolia* 5 wk after exposure to acute gamma radiation at doses of 25 Gy, 50 Gy and 100 Gy, the two higher doses resulted in decreases in the height, number of leaves and number of shoots. This was similar to the findings of Tangpong et al. (2009) with plantlets of *A. congensis* that had been exposed to acute gamma radiation at doses of 0 Gy, 20 Gy, 40 Gy, 60 Gy, 80 Gy and 100 Gy (at a dose rate of 264 Gy/h), where the number of new shoots, number of roots and number of leaves decreased as the dose increased. Chen et al. (2011) reported that *in vitro* callus culture of *Zoysia matrella* L. after 6 wk exposure to gamma irradiation at doses of 0 Gy, 5 Gy, 10 Gy, 20 Gy, 40 Gy, 80 Gy, 100 Gy, 150 Gy, 200 Gy, 250 Gy and 300 Gy, resulted in the highest callus regeneration rate and regeneration capacity after treatment with 20 Gy, with values of 27.08% and 91.67%, respectively. Furthermore, when the irradiation dose was increased to 100 Gy, 10.42% of the calli developed shoots but at 150 Gy both the regeneration rate and regeneration capacity declined significantly. In addition, Sianipar et al. (2013) with *in vitro* culture of *T. flagelliforme* Lodd. after 6 wk exposure to gamma irradiation at doses of 0 Gy, 10 Gy, 20 Gy, 30 Gy, 40 Gy and 50 Gy, reported significant differences between the normal mother plant (0 Gy), 20 Gy and 30 Gy with average plant heights of 9.57 cm, 3.41 cm and 2.43 cm, respectively, and the average number of shoots produced was 7.85, 6.03 and 5.00, respectively. Lee et al. (2014) studied *in vitro* explants of *Acorus calamus* that had been subjected to gamma irradiation at doses of 0 Gy, 20 Gy, 50 Gy, 70 Gy, 100 Gy, 120 Gy, 150 Gy, 170 Gy, 200 Gy, 250 Gy, 300 Gy, 400 Gy and 500 Gy resulting in non-mutants having a similar plant height (53.6 cm) compared to the wild-type control (57.4 cm); however, the plant height of the dwarf mutant was much shorter (16.0 cm) than the wild-type control. The low radiation dose caused the cells to stop growing; however, this did not cause cell death as the cells also have the ability to repair themselves, though if self-repair fails, this can result in mutations (Lamseejan, 1997; Wongpiyasatid, 2007).

Effect of chronic gamma irradiation of Anubias nana in the M_1V_1 generation at 60 d after irradiation

Effect of chronic gamma irradiation on survival

At doses of 0 Gy, 26.88 Gy, 39.39 Gy, 66.22 Gy and 84.15 Gy (at a dose rate of 0.45 Gy/h), none of the samples died (Table 2). Similarly, Tangpong et al. (2009) reported no deaths caused by chronic gamma irradiation with *A. congensis* N.E. Brown at doses of 0 Gy, 14.34 Gy, 28.60 Gy, 31.24 Gy, 42.90 Gy, 51.16 Gy, 65.55 Gy, 82.42 Gy, 91.69 Gy, 105.99 Gy and 120.30 Gy (at a dose rate of 0.71 Gy/h) because the lower dose of radiation probably caused little damage to the plant's genetic material so that the cells could repair themselves

Table 2 Survival percentage and growth rate percentage of *Anubias nana* in the M_1V_1 generation at 60 d after chronic irradiation

Radiation dose (Gy)	Total number irradiated	Number of surviving plantlets	Plantlet survival (% of control)	Number of new plantlets	Growth rate (% of control)
0 (Control)	24	24	100.00 ± 0.00[a]	30	100.00 ± 0.00[a]
26.88	30	30	100.00 ± 0.00[a]	37	100.30 ± 12.70[a]
39.39	29	29	100.00 ± 0.00[a]	36	99.73 ± 7.75[a]
66.22	33	33	100.00 ± 0.00[a]	39	95.83 ± 17.21[a]
84.15	32	32	100.00 ± 0.00[a]	37	94.12 ± 19.13[a]
105.52	24	22	91.67 ± 7.22[b]	24	81.82 ± 31.49[ab]
123.45	28	22	78.33 ± 2.89[c]	19	54.38 ± 6.81[b]
F test	-	-	**	-	*
C.V. (%)	-	-	3.07	-	18.52
$LSD_{(0.05)}$	-	-	5.14	-	29.02

C.V. = coefficient of variance; * = significant at 5% level; ** = significant at 1% level; data within columns followed by a common lowercase letter are not significantly different at the 1% and 5% level

and return to the normal cell cycle (Lamseejan, 1997). The plantlet survival percentage was not significantly ($p < 0.01$) different from the control at doses of 26.88–84.15 Gy. However, when the radiation dose increased to 105.52 Gy and 123.45 Gy, the plantlet survival percentage decreased to 91.67% and 78.33%, respectively (Table 2, Fig. 2). Kapare et al. (2017) reported similar observations for mutation induced by gamma irradiation in tissue culture of *Sesuvium portularcastrum* at low doses of 5 Gy, 10 Gy, 15 Gy, 20 Gy, 25 Gy, 30 Gy, 35 Gy and 40 Gy. The survival percentage of irradiated shoots reached 50% for a lethal value of gamma irradiation of 20 Gy and there was a significant ($p < 0.05$) percentage reduction in the survival rate of *in vitro* shoots with an increased gamma irradiation dose in the M_1V_1 generation.

Effect of chronic gamma irradiation on growth

At doses of 0 Gy–105.52 Gy, the growth rate percentages were not significantly ($p > 0.05$) different from the control. However, there was a significant ($p < 0.05$) difference at the highest radiation dose of 123.45 Gy. The growth rate percentage was different from that of other plants (Table 2). As the radiation dose increased, the growth percentage rate decreased (Fig. 2). This was similar to the results of Kapare et al. (2017) with *in vitro* shoot culture of *S. portularcastrum* exposure to gamma irradiation at low doses of 5 Gy, 10 Gy, 15 Gy, 20 Gy, 25 Gy, 30 Gy, 35 Gy and 40 Gy, where the maximum numbers of shoots were regenerated when exposed to 10 Gy of irradiation and non-irradiated shoot culture produced 3.88 multiple shoots, whereas

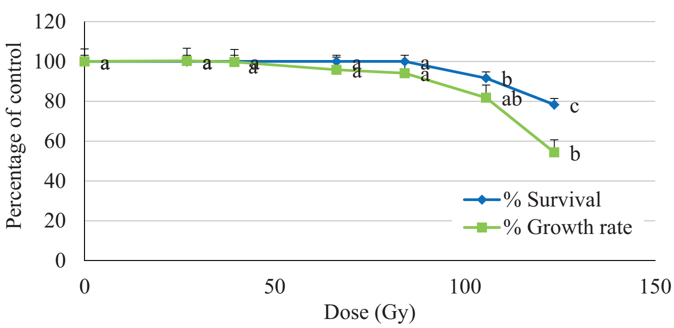

Fig. 2 Effects of radiation dose on survival percentage and growth percentage of *Anubias nana* plants in the M_1V_1 generation at 60 d after chronic irradiation

irradiated shoots at 10 Gy regenerated 6.6 shoots in 1 mth (M_1V_1 generation). As such, the results of the current study indicated that a high-gamma dose totally reduced regeneration of shoots, whereas low doses improved shoot regeneration 70% more compared to non-irradiated shoots. This was similar to the results reported by Tangpong et al. (2009) who studied the effect of chronic gamma irradiation on *A. congensis* N.E. Brown at doses of 0–120.30 Gy (at a dose rate of 0.71 Gy/h), as when the radiation dose increased to 105.99 Gy and 120.30 Gy, the number of shoots, number of leaves, leaf length and leaf width decreased. In the current study, the maximum dose used was 123.45 Gy and the growth rate percentage was 54.38% compared to non-irradiated plants; therefore, it was not possible to calculate the $GR_{50\,(60)}$ (Fig. 2).

Mutations observed in the M_1V_4 generation after acute gamma irradiation

The variation observed in the M_1V_4 generation samples of *A. nana* that were exposed to acute gamma irradiation at doses of 10 Gy, 20 Gy and 30 Gy produced mutations with frequencies of 38.46%, 49.04% and 31.25%, respectively (Table 3). A dose of 20 Gy produced the maximum mutation frequency of 49.04%, whereas *A. congensis* N.E. Brown had a maximum mutation frequency at a dose of 40 Gy (Tangpong et al., 2009). Lee et al. (2014) studied *in vitro* explants of *A. calamus* irradiated with gamma irradiation at doses of 0 Gy, 20 Gy, 50 Gy, 70 Gy, 100 Gy, 120 Gy, 150 Gy, 170 Gy, 200 Gy, 250 Gy, 300 Gy, 400 Gy and 500 Gy and reported that the dwarf mutant selected from the 150 Gy had a narrow leaf width (0.5 cm) compared to the wild-type control (1.2 cm). Non-mutants were similar to the wild type control in leaf width. In the current study, the most common mutation observed was dwarfism (24.04%) at a dose of 20 Gy. At a dose of 10 Gy, mutants had variegated leaves, dwarfism, albinism, light green leaves and abnormal leaves; at a dose of 20 Gy, mutants had variegated leaves, dwarfism, albinism and light green leaves; and at a dose of 30 Gy, mutants had dwarfism and light green leaves. However, at doses of 40–60 Gy, the plantlets turned yellow and finally died (Fig. 3). Overall, at every radiation dose, the most common characteristic was dwarfism (50.96%) as shown in Table 3.

Mutations observed in the M_1V_4 generation after chronic gamma irradiation

Variations were observed in the M_1V_4 generation samples of *A. nana* that were exposed to chronic gamma irradiation at doses of 26.88 Gy, 39.39 Gy, 66.22 Gy, 84.15 Gy, 105.52 Gy and 123.45 Gy with mutation frequencies of 36.60%, 19.12%, 48.97%, 49.12%, 55.43% and 55.83%, respectively (Table 4). Tangpong et al. (2009) reported that a maximum dose of 105.99 Gy with *A. congensis* N.E. Brown produced the maximum mutation frequency. In the current study, the most common mutation observed was dwarfism (25.44%) at a dose of 84.15 Gy (Table 4). At a dose of 39.39 Gy, mutants had variegated leaves, dwarfism and light green leaves; and at doses of 26.88, 66.22, 84.15, 105.52 and 123.45 Gy, all the mutant characteristics were present (variegated leaves, dwarfism, albinism, light green leaves and abnormal leaves) as shown in Table 4 and Fig. 4.

The appropriate dose of acute gamma irradiation at 60 d after irradiation to induce mutation for tissue culture of *A. nana* should be in the range 10–35 Gy (at a dose rate of 3.73 Gy/min). Plants that were exposed to 40 Gy of radiation or more turned yellow and eventually died. Variations were observed in the M_1V_4 generation such as variegated leaves, dwarfism, albinism, light green leaves and abnormal leaves. On the other hand, when *A. nana* plantlets were exposed to chronic gamma irradiation at 1.5 m from the gamma source (at a dose rate of 0.45 Gy/h), the appropriate dose at 60 d after irradiation to induce mutation for tissue culture of *A. nana* should be in the range 80–130 Gy. Variations were observed in the M_1V_4 generation such as variegated leaves, dwarfism, albinism, light green leaves and abnormal leaves.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Table 3 Mutation types and frequencies of *Anubias nana* in the M_1V_4 generation after acute gamma irradiation

Radiation dose (Gy)	Normal plantlet (%)	Mutation (%)	Mutant type				
			Variegated leaves	Dwarfism	Albinism	Light green leaves	Abnormal leaves
0 (Control)	100.00	0.00	0.00	0.00	0.00	0.00	0.00
10	61.54	38.46	3.21	14.42	1.60	14.42	4.81
20	50.96	49.04	6.73	24.04	0.96	17.31	0.00
30	68.75	31.25	0.00	12.50	0.00	18.75	0.00

Table 4 Mutation types and frequencies of *Anubias nana* in the M_1V_4 generation after chronic gamma irradiation

Radiation dose (Gy)	Normal plantlet (%)	Mutation (%)	Mutant type				
			Variegated leaves	Dwarfism	Albinism	Light green leaves	Abnormal leaves
0 (Control)	100.00	0.00	0.00	0.00	0.00	0.00	0.00
26.88	36.60	63.40	2.54	17.75	5.80	10.51	5.80
39.39	19.12	80.88	2.21	6.99	0.00	8.82	0.00
66.22	48.97	51.03	10.00	21.32	8.09	8.82	8.09
84.15	49.12	50.88	6.58	25.44	5.26	7.02	5.26
105.52	55.43	44.57	12.50	19.02	4.89	15.22	4.89
123.45	55.83	44.17	9.17	20.00	3.33	23.33	3.33

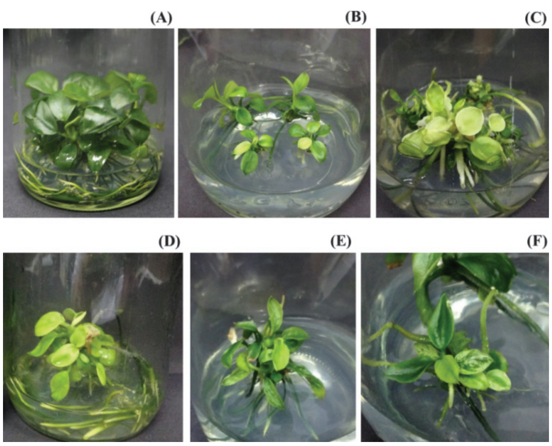

Fig. 3 Some mutants observed in the M_1V_4 generation after acute gamma irradiation: (A) control (0 Gy); (B) dwarfism; (C) albinism; (D) light green leaves; (E) abnormal leaves shape; (F) variegated leaves

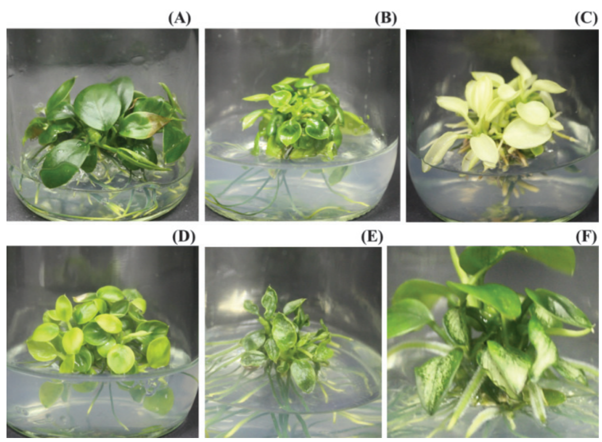

Fig. 4 Some mutants observed in the M_1V_4 generation after chronic gamma irradiation: (A) control (0 Gy); (B) dwarfism; (C) albinism; (D) light green leaves; (E) abnormal leaves shape; (F) variegated leaves

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