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In vitro mutagenesis of *Cymbidium* La bell "Anna Belle" by γ-rays irradiation and oligochitosan interaction

Duong Hoa Xo, Le Quang Luan*

Biotechnology Center of Ho Chi Minh City, No.2374 Highway, Trung My Tay Ward, District 12, Ho Chi Minh City, Vietnam

***Abstract*—** *The optimum media for multiplication of protocorm like bodies (PLBs) and shoot buds of Cymbidium La bell "Anna Belle" were studied in order to prepare the in vitro samples for irradiation. The values of LD_{50} (lethal dose of 50% samples) of PLBs, shoot buds and plantlets of tested Cymbidium after cultivation of 4 months were also determined about 35.0, 41.0 and 83.1 Gy, respectively. The addition of oligochitosan played as an very important trigger for promotion on the generation of shoot bud from PLBs after irradiation. The in vitro variations have been generated by γ-rays irradiation of PLBs with doses in range of 20 - 50 Gy. The highest mutant frequency (3.83‰) of C. La bell was found by the irradiation of PLB samples at 30 Gy. The different properties of obtained in vitro variations compared to wild types were found to be chlorophyll, short leaves, long leaves, and violet pericardium variations. The genetic relationships among generated variant lines in M_1V_4 and wild type were analyzed using RAPD techniques.*

Keywords*— *Cymbidium, in vitro propagation, irradiation, mutation, oligochitosan.

I. INTRODUCTION

Cymbidium is a genus of 50 species from Asia and they have attractive values in both art and commercialization [1]. *Cymbidium* La bell "Anna Belle" was imported to Vietnam several years ago and showed many good properties such as large number of flower shoots, big flower size, long life in vase, ease of plantation and high yield. Since *Cymbidium* is a very popular and favorite potted orchid in Asia, there have been several studies carried out for *in vitro* propagation of this genus using different methods. Brown el al. [2] studied on *in vitro* propagation of this orchid by seed and shoot tip culture method, while Nayak et al. [3,4] used shoot-tip, PLBs and thin cross section for cultivation. Other methods for micropropagation using callus [5,6] and embryo [7] were also established.

In addition, γ-rays irradiation technique in combination with tissue culture method had proven to be useful for mutation breeding and this technique has contributed towards improvements in agricultural crops and ornamental plants. According to the report of the joint FAO/IAEA programme for nuclear techniques in agriculture, there have been 3100 officially released mutant varieties from 170 different plant species in more than 60 countries. Among the mutant varieties, about 90% of these mutant varieties were generated by using radiation [8]. Several new flower varieties with high commercial value, such as chrysanthemum [9-12], anthurium [13], *Curcuma alismatifolia* [14], lily [15,16], etc. have been generated by γ-rays.

So far, most of cultivated *Cymbidium* varieties have been induced by crossing and the number of mutant orchids generated by radiation techniques is very few. For these reasons, the study aimed to optimize *in vitro* propagation conditions and generate *in vitro* mutagenesis of *Cymbidium* La bell "Anna Belle" by irradiation method combined with tissue culture techniques.

II. MATERIALS AND METHODS

Plant materials and chemicals

The orchid used in the present experiment namely *Cymbidium* La bell "Anna Belle" was supplied by Lang Biang Farm Ltd. This orchid grew in pots at an elevation of about 1500 m above sea level with an average temperature of about 18 ^oC and a range between 10 to 30 ^oC. N_6-benzyladenine (BA), indol-3-butyric acid (IBA), α-napthylacetic acid (NAA), thidiazuron (TDZ) and three kinds of medium namely Murashige and Skoog's (MS), Vacine & Went (VW) and Knudson C (KC) were supplied by Sigma-Aldrich Co. (St. Louis, Missouri USA). Oligochitosan with a molecular weight of about 16 kDa was prepared by the irradiation method as described previously [17].

Initial explant preparation

The new growths were taken from cymbidium pots at Lang Biang Farm, rinsed with tape water, and freed of dead, damaged, or excessively hard external tissues and parts. The shoots were then immersed in a mix of household bleach-distilled water (1:1; v/v). Excision of

explants was performed under a dissecting microscope on an open laboratory bench washed with 95% ethanol. External leaves and leaf primordial were removed to expose the shoot tips. Full-strength KC medium supplemented with coconut water (CW) (10%), sucrose (20 g/l) and activated charcoal (1 g/l) were used as the basic medium.

Protocorm like body proliferation

Protocorm like bodies (PLBs) with the size about 4 mm were cultured in MS, VW and KC media containing 10% CW, sucrose (20 g/l), 1 g/l charcoal and supplemented with BA or BA in combination with TDZ or NAA for PLBs multiplication. The number of PLBs, shoot buds and plantlets were determined after incubating in 30 days.

Shoot bud proliferation

For investigating the proliferation of shoot buds, PLBs with the size about 4 mm were cultured in Erlenmeyer flasks containing basic medium supplemented with BA, NAA or TDZ. The number of shoot buds per clump was determined after 6 weeks culture.

***In vitro* plantlet regeneration**

Individual shoot buds with 5 cm high and 3 expanded leaves detached from 6-week old shoot bud clumps were cultured in 250 ml Erlenmeyer flasks containing MS, VW and KC media with or without supplementation of 0.1 mg/l NAA and 10% CW. The shoot height and root length of *in vitro* plantlets were determined after culturing for 6 weeks.

Determination of LD_{50}

The radiosensitive tests for *in vitro* samples of the tested *Cymbidium* were established by irradiating PLBs, shoot buds and plantlets with γ-rays from a Co-60 source at various doses (five hundred of samples were applied for each dose) with a dose rate of 0.2 Gy/s. The survival rate of irradiated samples was determined after cultivation of 4 months for calculating the optimal dose for radiosensitivity, *i.e.* LD_{50} (lethal dosage of 50% irradiated samples) [14,18].

Variation induction

To generate *in vitro* variation, a thousand of PLB samples were irradiated by γ-rays at doses of 10, 20, 30, 40 and 50 Gy. The irradiated PLBs were then cultured in KC medium supplemented with 10% CW, sucrose (20 g/l), 1 g/l charcoal and 50 mg/l oligochitosan to generate the shoot bud. The culture medium was changed every 2 month for 12 months and the individual shoot buds with 3 - 4 expanded leaves generated from irradiated samples was detached for screening.

RAPD analysis

Total genomic DNA was extracted from frozen young leaves following the modified CTAB (cetyl trimethyl ammonium bromide) procedure described by Li et al. [19]. 100 mg frozen leaf tissue were ground to powder in liquid nitrogen using a mortar and pestle. The powder was transferred into 10 ml centrifuge tubes and was mixed with 3 ml of preheated (65 °C) 2X CTAB extraction buffer (100 mM Tris-HC1 (pH 8.0), 1.4 M NaC1, 20 mM EDTA (ethylene diamine tetra acetic acid), 2% CTAB, 2% (w/v) P-mercaptoethanol, 1% (w/v) polyvinylpyrrolidone (PVP40)). The mixture in the tubes was incubated at 65 °C for 30 min, then cooled and mixed with 500 µl tris-phenol, and held at 65°C for 15 min. The tubes were gently inverted upside down for several times and centrifuged at 4°C and 14,500 x g for 15 min. The supernatant was transferred to a 10 ml centrifuge tube and the same volume of chloroform was added. Then, incubation and centrifugation were repeated once. The supernatant was mixed with 0.7 volume of isopropanol, and then held at -20 °C for 30 min. The aqueous phase was discarded while the pellets were washed with 70% ethanol twice and absolute alcohol twice, then transferred to a 1.5 ml centrifuge tube, and dried at room temperature. The dried pellets were suspended in 600 µl HS-TE (10 mM Tris-HC1 (pH 8.0), 1 M NaCl, 0.1 mM EDTA (pH 8.0)), and then an equal volume of phenol/chloroform was added and centrifuged. The last procedure was repeated using chloroform replacing phenol/chloroform and centrifuged. The supernatant was uniformly mixed with 0.7 volume of isopropanol, and then held at -20 °C for 1 h. The mixture was centrifuged and the pellets were washed with 70% ethanol and absolute alcohol, respectively, and then dried at room temperature. The dried pellets were precipitated in 100 µl TE [100 mM Tris-HC1 (pH 8.0); 1 mM EDTA (pH 8.0)]. RNA was removed by RNaseA (100 µg ml^{-1}) for 1 h at 37 °C. The yield of DNA per gram of leaf tissue extracted was measured using a BioPhotometer (Eppendorf) spectrophotometer a 260 nm. The DNA purity was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm and the quality was evaluated by 0.8% agarose gel (0.5 µg/ml EB) electrophoresis. Working solutions of the DNA were prepared by diluting the stocks at 10 ng/µl in sterile distilled water.

The RAPD reactions were performed following the procedures described by Li, et al. [19]. A total volume of 25 µl containing: 25 ng of template DNA, 0.2 µM of 20 decamer oligonucleotide primers (OPA and OPD from 01 to 10) (Sigma), 1.0 U Taq DNA polymerase (Takara, Japan), 0.2 mM each *dNTP* (Sigma, Molecular biological grade), 2 mM $MgCl_2$, 10 mM Tris-HC1 (pH 8.3), 50 mM KC1 (pH 8.3), and sterilized water. DNA amplification was carried out in Mastercycler gradient (Eppendorf) and the thermal cycling programme was as follows: An initial denaturation cycle of 3 min at 94ºC, followed by 40 cycles comprising of 1 min at 94ºC, 1.5 min at 37ºC, and 2.5 min at 72°C; 72°C for 5 min was used for the final extension. The amplification products were separated by electrophoresis in 1.5% agars gels in 0.5X TBE at 150 V.

Each amplification reaction was repeated at least twice. RAPD polymorphic bands were visualized and photographed using ultraviolet illumination, and were scored as present (1) or absent (0). The data were analyzed using the SIMQUAL (similarity for qualitative data) routine to generate Dice similarity coefficients and these similarity coefficients were used to construct dendrograms using the NTSYSpc (ver.2.10) program.

Incubational conditions and statistical analysis

The pH of media was adjusted to 5.8 by KOH and HCl before autoclaving at 121 ^{o}C and 105 kpa for 15 min. Cultures were incubated in a cultural room at 25 ± 1 ^{o}C under 16-h photoperiod provided by fluorescent lamp at 45 µmol m^{-2} s^{-1} photosynthetic photon density flux. All experiments were repeated three times. Data were statistically analyzed by variance analysis (ANOVA) and means were compared using the least significant difference (LSD) at 5% probability level and standard deviation Duncan's multiple range test [20].

III. RESULTS

PLB and shoot bud multiplication

Protocorm like bodies (PLBs) with the diameter about 4 mm and shoot buds with 3 cm high and 2 expanded leaves were used for PLBs and shoot buds multiplication, respectively. The results in Table 1 showed that the supplementation with 2.0 mg/l BA displayed the best effect on the formation of PLBs of *C* La bell (10.29 PLBs per samples). In addition to, the supplementation of BA (2.0 mg/l) combined with 0.1 - 0.2 mg/l TDZ or NAA stimulated the formation of PLBs.

On the other hand, the results from Table 2 showed that the highest shoot proliferation rate (8.25 - 8.29 shoot buds per sample) was obtained by the supplementation of BA at 0.2 mg/l. For shoot bud proliferation of mentioned *Cymbidium*, number of shoot buds generated from PLB cultured on MS or KC media after 6 weeks incubation was higher than that of sample culture on VW medium.

Plantlet regeneration

The shoot buds with 5 cm high and 3 expanded leaves were used as starting materials for plantlet regeneration. The results from Table 3 showed that the supplementation of NAA (0.1 mg/l) and coconut water (10%) stimulated the growth of *Cymbidium* plantlets incubated on all of three media (MS, KC and VW) and the best suitable media were found to be MS and VW.

Effect of γ-rays on the growth of *in vitro* samples

To investigate the LD_{50} (the lethal dose of 50% samples) of *in vitro Cymbidium* samples, the PLBs with the diameter about 4 mm, shoot buds with 3 cm high and 2 expanded leaves or plantlets with good development were used for irradiation. The results from Fig. 1 indicated that LD_{50} of PLBs and shoot buds of *C.* La bell after cultivation of 4 months were 35.0 and 41.0 Gy, respectively, while the LD_{50} value of plantlets was determined about 83.1 Gy after cultivation 4 months in a greenhouse.

Induction and screening *in vitro* variation lines with oligochitosan association

A thousand PLBs were irradiated at the dose range of 20 - 50 Gy and then cultured on basic medium containing 0.2 mg/l BA with or without supplementation of oligochitosan. It can be seen from Table 4 that, the survival samples after irradiation developed very low in the medium without supplementation of oligochitosan and the number of generated shoot bud was quite low (see Fig. 2) even for samples irradiated at a low dose (10 Gy). In contract, the irradiated PLBs cultured in medium supplemented with oligochitosan generated a large number of shoot bud after 12 month even for samples irradiated at 50 Gy.

The results from Table 4 also showed that the variation was not induce from sample irradiated at 10 Gy, while the total frequencies of *in vitro* variation of *C.* La bell generated from the samples irradiated at 20, 30, 40 and 50 Gy were found about 1.60, 3.83, 3.15 and 2.27‰, respectively. The highest variation frequency of *C.* La bell was obtained by the treatment of 30 Gy. The variations of mentioned *Cymbidium* orchids generated by γ-rays irradiation mainly belong to four types as follow (see Fig. 3): Chlorophyll variations, short leaves variations (leaves become shorter), long leaves variations (the leaves become longer and bigger) and violet pericardium variations (the color of pericardium become violet).

RAPD analysis

Among the screened variations, 3 stable variant lines screened in M_1V_4 (CLB-20Gy-1.1: Large leave variation, CLB-20Gy-2.1: Short leave variation and CLB-20Gy-3.1: Chlorophyll variation) were analyzed by RADP technique using 20 decamer oligonucleotide primers (OPA and OPA from 01 to 10). 7 primers (OPA 01, OPA 10, OPD 02, OPD 05, OPD 06, OPD 07 and OPD 08) were found to generate polymorphic amplified fragments with an average polymorphic ratio approximately 86.8% (Table 5). The scored applified data are presented in Fig. 5 and similarly matrix in Table 6. It can be seen that the genetic relationships of wild type (CLB-ctrl) and variation lines namley CLB-20Gy-1.1 CLB-20Gy-2.1 CLB-20Gy-3.1 were found at 80, 46.7 and 60%, respectively, while the genetic relationship among mentioned variation lines was calculated from 53.3 to 66.7%. The phylogenetic tree (Fig 5) indicated that the group of mutants is different from the other one with only the control.

IV. DISCUSSION

Optimization of *in vitro* cultural condition

6-benzylaminopurine (BA) is a good plant hormone for PLBs multiplication. In this study, BA displayed the best effect on the formation of PLBs of *C.* La bell (10.29 PLBs per samples and the obtained results are in good agreement with that of Najak et al. [4]. The addition of 2.0 mg/l BA combined with NAA or TDZ at concentrations of 0.1 and 0.2 mg/l displayed a stronger effect on the PLB multiplication compared with that of a single addition of BA. This medium is very important for preparation of a large number of PLBs of this variety for irradiation.

In addition to, BA, TDZ and NAA have been widely used for proliferation of many kinds of plant *in vitro*. The results from Table 2 showed that the supplementation of the mentioned plant hormones increased the shoot proliferation rate of the tested orchid. It can be seen that BA had a higher effect on stimulation of shoot proliferation rate compared to those of TDZ and NAA, specially, the highest stimulation effect (8.25 - 8.29 shoot buds per clump) was found by the supplementation of BA at 0.2 mg/l and the obtained results are in good agreement with results on *C aliofolium* (L.) Sw. of Najak et al. [3]. On the other hands, for shoot bud proliferation of mentioned *Cymbidium*, the usage of MS and KC mediums were found to be better than that of VW. Therefore, the MS medium supplemented with 0.2 mgl BA was selected as the best suitable medium for shoot bud generation of irradiated samples.

The plantlet regeneration is very important step in tissue culture technique to induce the *in vitro* plantlets. In present study, the plantlets were induce for two purposes, first was induce the materials for irradiation and the second was induce plantlets from the shoot clusters of irradiated samples. It was found that the supplementation of NAA (0.1 mg/l) and 10% coconut water (CW) stimulated the growth of *Cymbidium* plantlets incubated on three kinds of mediums (MS, KC and VW) and the best suitable mediums were found to be MS and VW mediums. The supplementation with CW (10%) and NAA (0.1 mg/l) was optimum for plantlet regeneration of tested *Cymbidium* variety.

Thus, the completed process of *in vitro* propagation including initial explant preparation, PLB multiplication, shoot tip generation and plantlet regeneration of *Cymbidium* La bell "Anna Belle" has been built up. This process was used for preparation samples for irradiation and cultivation of the survival samples collected after irradiation.

Generation of variation lines by γ-rays irradiation

Among thousands of mutant varieties have been generated by radiation methods, ornamental and decorative plants occupied about 25% [8,21]. γ-rays reported as useful mutagens for plant mutation breeding and it was employed to develop 64% of the radiation-induced mutant varieties [9,10-16,21]. Kozlowska-kalisz [22] reported that, a dose of 20 Gy inhibited growth of samples and 70 Gy was the lethal dose of PLBs of the *Cymbidium* orchid. For generation of mutation of plants by γ-rays irradiation, the investigation of the radiosensitivity test, *i.e.* lethal dose of 50% samples (LD_{50}) is important for plant mutation breeding studies to focusing the effective doses at which frequency the mutants will be high obtained [9,13,22-25]. In this study, five hundred of *in vitro* samples (PLBs, shoot buds or plantlets) of *C.* La bell were applied for each irradiation dose. The results from Fig. 1 indicated that LD_{50} of PLBs and shoot bud after cultivation of 4 months were 35.0 and 41.0 Gy respectively, while the LD_{50} value of plantlets was determined about 83.1 Gy after cultivation 4 months in a greenhouse.

After obtaining the LD_{50}, A thousand of PLBs of mentioned orchid were applied for γ-rays irradiation at doses of 10, 20, 30, 40 and 50 Gy for mutant generation. Samples after irradiation were then cultured on basic media containing 0.2 mg/l BA with or without supplementation of oligochitosan. It can be seen from Table 4 that, the survival samples after irradiation developed very low in the medium without supplementation of oligochitosan and the number of generated shoot bud was quite low (see Fig. 2) even for samples irradiated at a low dose (10 Gy). There is no variation is found from irradiated samples incubated in medium without oligochitosan supplementation. The reason may due to the number of generated shoot bud is small. In contract, the irradiated PLBs cultured in medium supplemented with oligochitosan generated a large number of shoot bud after 12 month even for samples irradiated at 50 Gy. Oligochitosan has been proved to have several novel features such as growth promotion for *in vitro* flower plant samples and increase of the survival rate of plantlets acclimatized in a greenhouse [17], induction of antibiotic phytoalexins to prevent infection from fungal diseases [26,27], reduction of the damage caused by toxic elements (zinc, vanadium etc.) [28] and enhancement of the seed germination rates [29]. Recently, we also found that oligochitosan promoted the generation of shoot bud from PLBs of slipper orchids (*Paphiopedilum callosum* and *Paphiopedilum delenatii*) after irradiation by 320 MeV $^{12}C^{6+}$ ion-beams accelerated with an AVF cyclotron [30].

γ-rays irradiation can induce mutants in numerous plants such as chrysanthemum [12,13,25,31]. Mutation breeding by application of *in vitro* techniques in combination with gamma rays irradiation method has been successful with several flower plants such as chrysanthemum [32,33] and slipper orchids [30], and so on. Even so, the use of radiation for mutation breeding of *Cymbidium* orchids is still limited.

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The results from Table 4 also showed that the variation was not induce from sample irradiated at 10 Gy, while the total frequencies of *in vitro* variation of *C.* La bell generated from the samples irradiated at 20, 30, 40 and 50 Gy were found about 1.60, 3.83, 3.15 and 2.27‰, respectively. The highest variation frequency of *C.* La bell was obtained by the treatment of 30 Gy. The variations of mentioned *Cymbidium* orchids generated by γ-rays irradiation mainly belong to four types as follow (see Fig. 3): Chlorophyll variation, short leaves mutants (leaves become shorter), long leaves variation (the leaves become longer and bigger) and violet pericardium variation (the color of pericardium become violet). Among the screened variations, the variations with the color change of pericardium into violet color (violet pericardium variations) and the variations changes in leaf size (the leaves become longer or shorter) were found from samples irradiated from 20 to 50 Gy, while the chlorophyll variations were only found by the samples irradiated at lower doses (20 – 40 Gy). In previous report [30], the *in vitro* mutant types of *P. callosum* and *P. delenatii* such as chlorophyll mutants and leaf size mutants were generated by ionization irradiation of PLBs.

RAPD analysis

The usage of random amplified polymorphic DNA (RAPD) markers to detect the genetic relationships of orchid has been reported and it was found that the RAPD is a convenient technique for detecting the difference in DNA among *Cymbidium* cultivars [19,34]. Puchooa et al. [13] also used RAPD technique for the analysis of the change in DNA of mutant anthurium generated by gamma radiation. In addition, changes in DNA caused by chemical agent treatment of mutant *Senna occidentalis* [35] and mutant *Helianthus annuus* [36] resulted in genetic variation were successfully detected using RAPD technique. On the other hand, Shin et al. [37] also used the same technique for analyzing the genetic relationship between mutant sweet potato and wild type plant. In our previous report, the chande in DNA of *P. delenatii* and *P. callosum* mutant lines induced by ion beams was also successful analyzed by RADP using ODP decamer oligonucleotide primers. In present study, we also used RAPD with 7 selected ODP and OPA primers for analyzing the genetic relationships of wild type of tested *Cymbidium* variety and 3 selected mutant lines induced from samples irradiated at 30 Gy (CLB-30Gy-1.1, CLB-30Gy-2.1 and CLB-30Gy-3.1). All of the 7 selected primers induced polymorphic amplified fragments with a high polymorphic ratio (86.8%). The low genetic relationships between 3 variation lines (CLB-30Gy-1.1, CLB-30Gy-2.1 and CLB-30Gy-3.1) and of wild type (CLB-ctrl) indicated the changes in DNA of mutant lines by ion-beams. Our results are in agreement with the findings of Puchooa et al. [13] and Mostafa [36]. Since genetic relationships among 3 mutant lines and of wild type were rather low (46.7 – 80.0%), the phylogenetic tree grouped the mutants and the control into different clusters. The mutant lines were scored in one cluster, while the control was scored in the others. Thus, changes in DNA induced by γ-rays irradiation and resultant genetic variation in *C.* La bell "Anna Belle" mutant lines can be rapidly detected by RAPD analysis. Our results are in agreement with our previous results [30] and those of previous studies using RAPD for analyzing the changes in DNA of mutant plants [13,35-37].

V. CONCLUSIONS

The optimum conditions for PLBs multiplication, shoot bud proliferation and plantlet regeneration were found to be KC medium supplementation 2.0 mg/l BA in combination with 0.1 mgl NAA, KC medium supplementation 2.0 mg/l BA, and MS medium supplementation with 0.1 mg/l NAA, respectively. The values of LD_{50} of PLBs, shoot buds and plantlets of *C* La bell "Anna Belle" after cultivation of 4 months have been determined. To generate the mutants of the mentioned orchid by γ-rays irradiation, PLBs were suitable samples for irradiation and the supplementation of oligochitosan was very important for generating the shoot buds from irradiated sample of this Cymbidium. γ-rays irradiation at doses from 20-50 Gy induced *in vitro* mutants of Cymbidium orchid and the suitable dose was found at 30 Gy. The screened mutant lines provide promising materials for developing new mutant varieties for *C* La bell "Anna Belle".

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Table.1: Effect of phytohormone onto the PLB multiplication rate of C. La bell "Anna Belle"

Plant hormone, mg/l			PLBs per explant[x]
BA	NAA	TDZ	
0	0	0	4.04[a]
0.5	0	0	6.70[b]
1.0	0	0	8.15[c]
2.0	0	0	10.29[e]
3.0	0	0	9.45[d]
5.0	0	0	9.11[d]
10	0	0	9.36[d]
2,0	0.1	0	12.12[g]
2,0	0.2	0	11.58[f]
2,0	0.3	0	10.55[e]
2,0	0.5	0	9.80[d]
2,0	1.0	0	9.12[d]
2,0	0	0.1	11.15[f]
2,0	0	0.2	11.08[e]
2,0	0	0.3	10.48[e]
2,0	0	0.5	10.14[e]
2,0	0	1.0	9.42[d]

[x] Mean values followed by the same letter within a column are not statistically different according to a Duncan's multiple range test at $P < 0.05$.

Table.2: Effect of plant hormone on shoot bud proliferation for C. La bell "Anna Belle"

Plant hormone	Conc., mg/l	Shoot bud proliferation		
		MS	VW	KC
Control		4.40[a]	4.68[c]	4.70[b]
BA	0.1	5.51[b]	5.69[c]	5.70[e]
	0.2	8.25[c]	8.07[e]	8.29[f]
	0.3	6.13[b]	5.39[d]	6.18[d]
	0.5	4.18[a]	4.40[b]	5.07[c]
TDZ	0.1	4.16[a]	4.15[b]	4.33[a]
	0.2	3.84[a]	4.35[b]	4.24[a]
	0.3	3.79[a]	4.24[b]	4.02[a]
	0.5	3.41[a]	3.55[a]	3.78[a]

NAA	0.1	4.81[a]	4.88[c]	4.90[b]
	0.2	4.72[a]	4.63[c]	4.73[b]
	0.3	4.13[a]	4.75[c]	4.77[b]
	0.5	3.92 [a]	4.19[b]	4.23[a]

Mean values followed by the same letter within a column are not statistically different according to a Duncan's multiple range test at $P < 0.05$.

Table.3: Plantlet generation of C. La bell "Anna Belle"

Medium	NAA, mg/l	CW, %	Shoot hight, cm	Root length, cm
MS	0	0	7.77 ± 0.11	1.8 ± 0.13
	0.1	0	8.02 ± 0.11	2.4 ± 0.10
	0.1	10	8.49 ± 0.10	3.3 ± 0.11
KC	0	0	7.34 ± 0.14	1.9 ± 0.10
	0.1	0	7.60 ± 0.17	2.5 ± 0.12
	0.1	10	8.14 ± 0.09	3.0 ± 0.11
VW	0	0	7.51 ± 0.22	1.9 ± 0.13
	0.1	0	8.10 ± 0.19	2.1 ± 0.10
	0.1	10	8.43 ± 0.20	3.1 ± 0.11

Table.4: The in vitro variation types of C. La bell "Anna Belle" generated by γ-rays

Dose, Gy	OC concentration, mg/l	Number of shoot bud generation	Type variation	Number of variation	Frequency, ‰
10	0	1520	-	0	0
	50	20000	-	0	0
20	0	1041	-	0	0
	50	19128	*- Chlorophyll variation*	*3*	*0.15*
			- Short leaves variation	*7*	*0.36*
			- Long leaves variation	*19*	*0.99*
			- Violet pericardium variation	*2*	*0.10*
			Total	21	1.60
30	0	478	-	0	0
	50	15952	*- Chlorophyll variation*	*2*	*0.13*
			- Short leaves variation	*38*	*2.39*
			- Long leaves variation	*20*	*1.25*
			- Violet pericardium variation	*1*	*0.06*
			Total	61	3.83
40	0	231	-	0	0
	50	16142	*- Chlorophyll variation*	*3*	*0.17*
			- Short leave variation	*29*	*1.60*
			- Long leave variation	*21*	*1.16*
			- Violet pericardium variation	*4*	*0.22*
			Total	57	3.15
50	0	129	-	0	0
	100	3965	*- Short leave variation*	*2*	*0.50*
			- Long leave variation	*4*	*1.01*
			- Violet pericardium variation	*3*	*0.76*
			Total	9	2.27

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Table.5: The sequences of 7 selected RAPD primers and the amplification results on C. La bell "Anna Belle"

Primer No.	Primer code	Sequences (5' → 3')	Total amplified fragments (a)	Polymorphic amplified fragments (b)	Polymorphic ratio (b/a x 100), %
1	OPA 01	CAGGCCCTTC	7	5	71.4
2	OPA 10	GTGATCGCAG	12	12	100
3	OPD 02	GGACCCAACC	10	9	90.0
4	OPD 05	TGAGCGGACA	11	10	90.9
5	OPD 06	ACCTGAACGG	11	7	63.6
6	OPD 07	TTGGCACGGG	13	13	100
7	OPD 08	GTGTGCCCCA	12	11	91,7
Sum			64	67	
Mean			10.7	9.6	86.8

Table.6: Percent of similarity matrix from wild type C. La bell "Anna Belle" and 3 selected variation lines

	CLB-ctrl	CLB-30Gy-1.1	CLB-30Gy-2.1	CLB-30Gy-3.1
CLB-ctrl	100			
CLB-30Gy-1.1	80.0	100.0		
CLB-30Gy-2.1	46.7	60.0	100.0	
CLB-30Gy-3.1	60.0	66.7	53.3	100

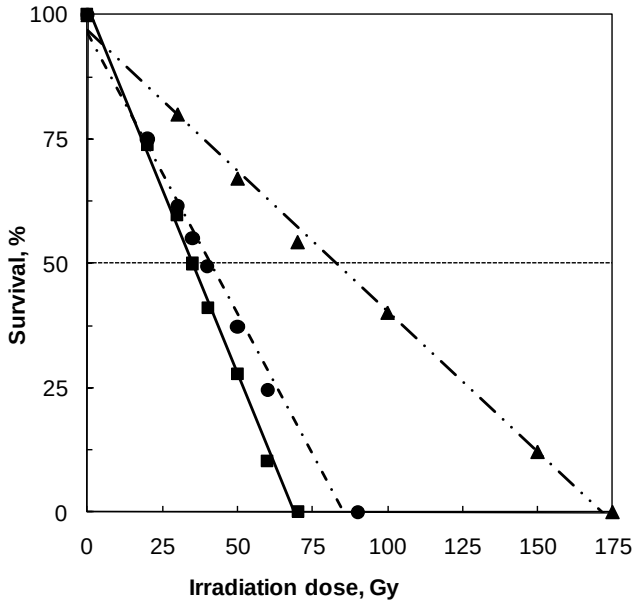

Fig.1: Survival ratio of Cymbidium La bell "Anna Belle" samples irradiated by γ-rays after cultivation of 4 months. (■): PLBs, (●): shoots buds and (▲): plantlets

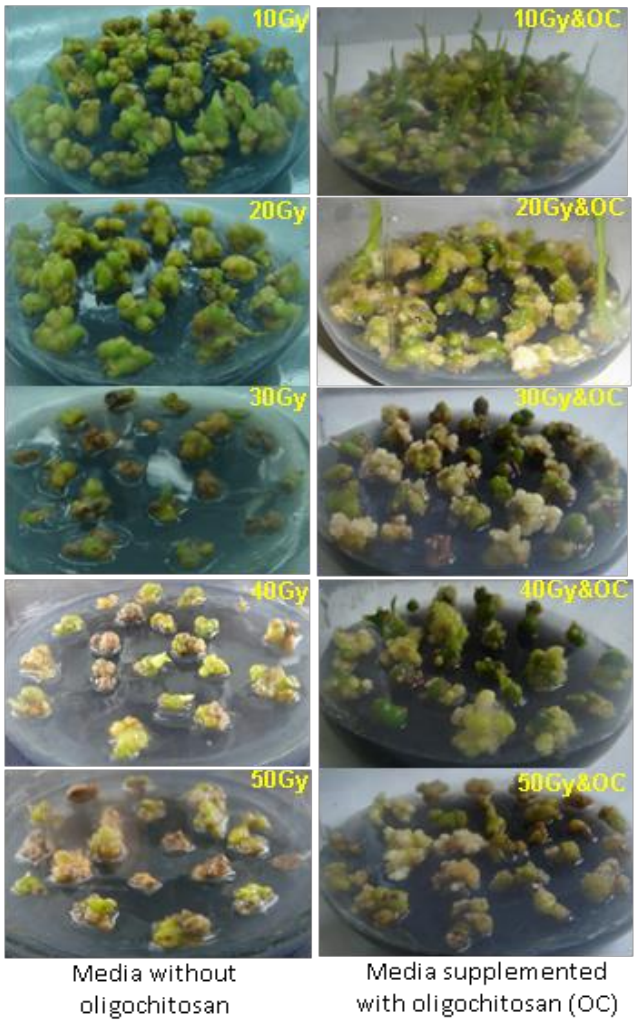

Fig.2: The development of PLBs after irradiation at various doses and cultured on media supplemented with and without oligochitosan

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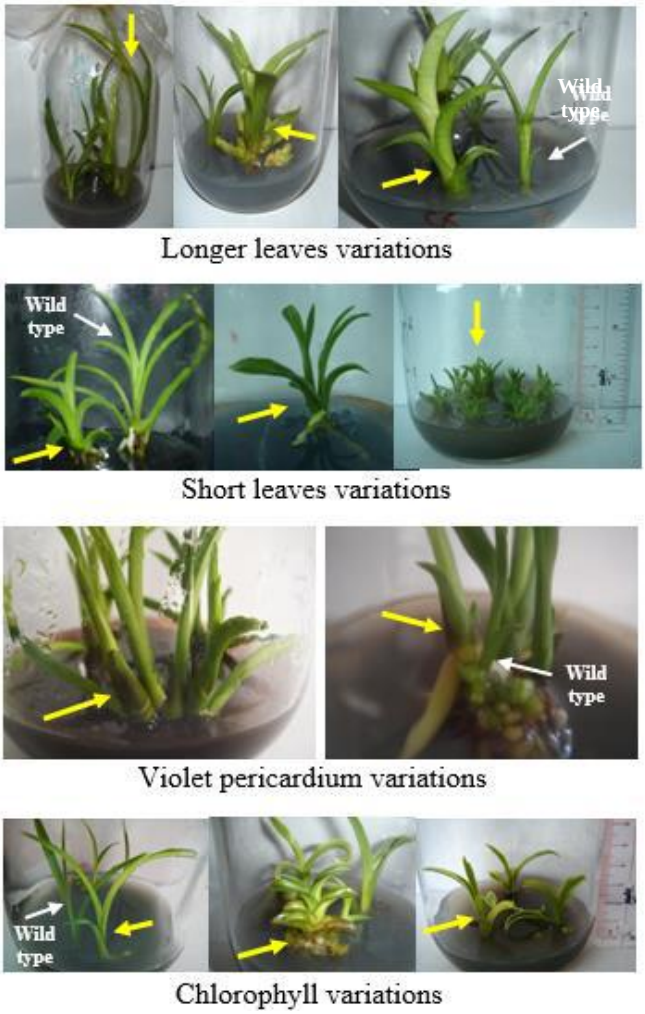

Fig.3: The in vitro variation types of Cymbidium La bell "Anna Belle" generated by γ-rays irradiation

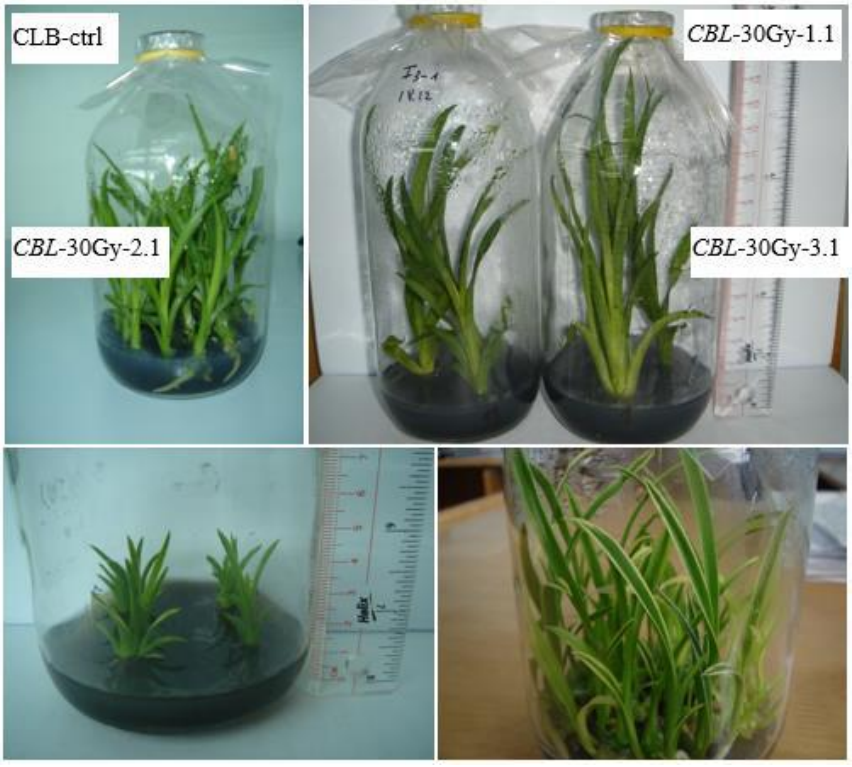

Fig.4: The stable variant lines of Cymbidium La bell "Anna Belle" generated by γ-rays irradiation at 30 Gy. CLB-ctrl: Wide type, CLB-20Gy-1.1: Large leave variation, CLB-20Gy-2.1: Short leave variation, and CLB-20Gy-3.1: Chlorophyll variation

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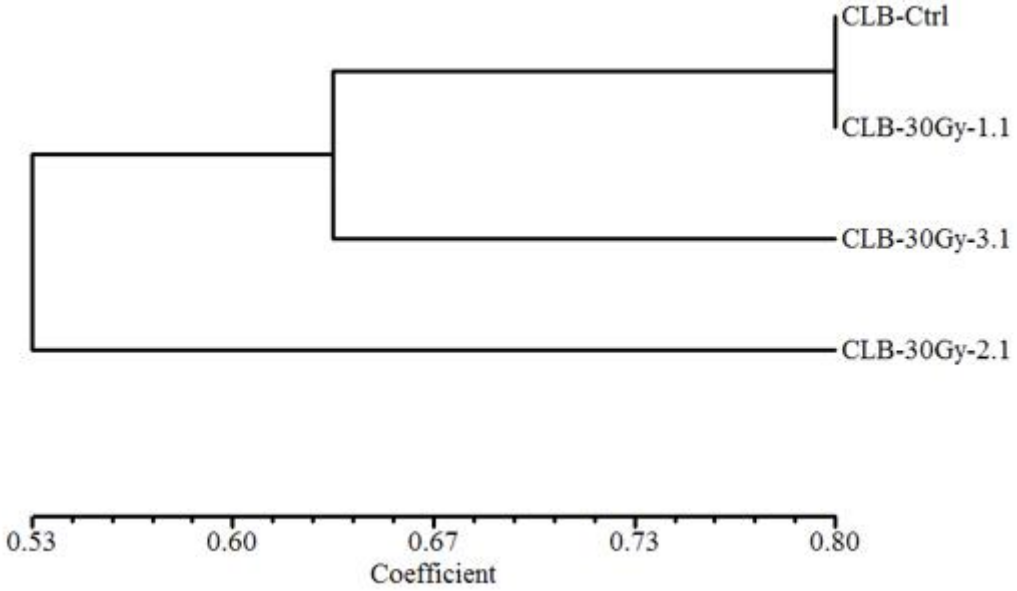

Fig.5: Dendrogram of the genetic relationship between the wild type and 3 selected variation lines of C. La bell "Anna Belle"