Induced *in vitro* Mutagenesis of Aquatic Plant *Cryptocoryne willisii* Engler ex Baum Using Gamma Irradiation to Develop New Varieties

NORHANIZAN, S.[1/,3/], NURAINI, A. W.[3/], ROFINA YASMIN, O.[2/,3/] and NORZULAANI, K.[2/,3/]

[1/]*Freshwater Fisheries Research Division, FRI Glami Lemi, Department of Fisheries, Jelebu, 71650 Negeri Sembilan, Malaysia*
[2/]*Centre for Research in Biotechnology for Agriculture (CEBAR), University of Malaya, 50603 Kuala Lumpur*
[3/]*Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur*

Abstract: Two new varieties of *Cryptocoryne willisii* were developed through mutagenesis in this work, where shoot-tip explants of *C. willisii* were subjected to a range of ^{60}Co gamma ray irradiations: (0, 100, 200, 300, 400, 500, 600, 700 and 800 Gray). The LD_{50} for the tissue culture plants of *C. willisii* was at 25 Gy, which was considered as an appropriate dosage to induced mutations in this plant. About two thousand shoot-tip explants were irradiated at 25 Gy and variants from the M_1, M_2, M_3 and M_4 generations were screened for morphological differences. The shoots were subcultured repeatedly until the 4th generation (M_4) to ensure stability of mutants. Although initially many regenerants with different morphological traits were produced, only two mutants remained stable. The mutants obtained were dwarf plants (D1) and plants of taller stature with pigmented leaves (G1) in comparison to control cultures. This was verified from the significant F value from the ANOVA test, where $P<0.05$. The Inter-Retrotransposon Amplified Polymorphism (IRAP) markers were used to distinguish the D1 and G1 genomes from normal *C. willisii* genomes. The analysis revealed two specific bands 325 bp and 420 bp using Nikita primer for the D1 mutant and 240 bp and 300 bp using combination of 3'LTR primer and LTR 6149 primer for the G1 mutant.

Keywords: *Cryptocoryne willisii*, gamma irradiation, inter- retrotransposon analysis polymorphism analysis (IRAP)

Abstrak: Dua variasi baru *Cryptocoryne willisii* teleh dibangunkan melalui mutagenesis dalam kajian ini, di mana eksplan pucuk *C. willisii* telah didedahkankepadasinaran gamma ^{60}Co yang berbeza: (0, 100, 200, 300, 400, 500, 600, 700 and 800 Gray). Dos kematian 50% (LD_{50}) untuktumbuhan tisu kultur *C. willisii* ialah pada dos 25 Gray. Dan oleh itu, nilai dos tersebut telah digunakan untuk mengakibatkan mutasi pada tumbuhan. Lebih kurang dua ribu eksplan pucuk telah diradiasi pada 25 Gy dan varian-varian dari generasi M_1, M_2, M_3 and M_4 telah ditapis untuk perubahan morfologi. Pucuk-pucuk tersebut telah disubkultur berulang kali sehingga generasi ke-4 (M_4) bagi memastikan kestabilan mutan. Walaupun pada peringkat awal banyak regeneran dengan sifat-sifat morfologi yang berbeza terhasil, namun hanya dua sahaja mutan yang stabil. Mutan yang terhasil adalah tumbuhan kerdil (D1) dan tumbuhan tinggi dengan daun berpigmentasi (G1) berbanding dengan kultur kawalan. Ini disahkan oleh nilai F signifikan dari ujian ANOVA, di mana $P<0.05$. Penanda Inter-Retrotransposon Amplified Polymorphism (IRAP) telah digunakan untuk pengesahan genom D1 dan G1 dari normal genom *C. willisii*. Analisis tersebut telah dapat menunjukkan dua jalur spesifik 325 bp dan 420 bp dengan menggunakan primer Nikita untuk mutan D1 dan untuk G1, dua jalur spesifik adalah 240 bp dan 300 bp dengan menggunakan primer 3'LTR primer dan LTR 6149.

Introduction

Cryptocoryne willisii Engler ex Baum belongs to the family Araceae, genus *Cryptocoryne Fischer* ex Wydl is commonly distributed in Sri Lanka. Its synonyms are *C. pseudo-beckettii* (De Wit, 1964), *C. axelrodii* Rataj (Rataj, 1977) and *C. undulata* Wendt (Rataj, 1977).

C. willisii is a small aquatic plant, originated from Sri Lanka. In nature, this plant propagates through runners. The substrate for planting in aquarium is plain washed gravel, with lighting from moderate to bright white light, at pH 6.8-7.2 (aquarium water), and water hardness at 3-8°dH. The ideal water temperature for the growth of *Cryptocoryne* is around 20-26°C. In aquarium, *C. willisii* can grow up to 20 cm height. The morphology of the stemmed leaves is oblong-oval to lanceolate and green in colour with brownish to green stems.

C. willisii seldom flowers and during seasonal flowering it produces non viable seeds when grown in natural habitat. (Rataj, 1977; Windelov, 1987; Cook, 1996). Thus, production of new plant variety

through cross breeding between species is not possible. However, *C. willisii* is one of the most popular aquarium plants in the world and a novel variety is very much sought for in order to be competitive in this aquarium business (Hiscock, 2005). In this paper, novel mutants of *C. willisii* were obtained through gamma irradiation.

Mutants were distinguished by the morphological differences from normal plants. Besides that, mutants were also distinguished by using molecular marker technique. Recently, more advance molecular marker techniques are used in mutation study. Advance molecular marker techniques are the combination of several advantages of basic techniques and incorporated with modification in the methodology to gain better result of the genetic material. The among advance techniques are the transposable elements-based molecular marker andretrotransposon-based molecular marker including (1) Inter-retrotransposon amplified polymorphism (IRAP) and Retrotransposon-microsatellite amplified polymorphism (REMAP), (2) sequence-specific amplification polymorphism (S-SAP), (3) Retrotransposon-based insertion polymorphism (RBIP).

IRAP analysis was firstly described by Kalendar *et al.* (1999). IRAP is based on the PCR amplification of genomic DNA fragments, which lie between two-retrotransposon insertion sites. Polymorphism is detected by the presence or absence of the PCR product. Lack of amplification indicates the absence of the retrotransposon at the particular locus. The technique was originally developed using the BARE-1 retrotransposon, which is present in the barley genome in numerous copies. About thirty bands were visualized by a single PCR reaction (Kalendar *et al.*, 1999). IRAP markers were extremely polymorphic, which made them useful for evaluating intraspecific relationships, investigating linkage, evolution, determination of varieties and mutants and genetics diversity in plants (Dariusz, 2006).

The objective of this study is to develop new variety of *C. willisii* by using gamma irradiation.

Materials and Methods

Plant material and growth conditions

Plants of *C. willisii,* received from Freshwater Fisheries Research Centre, Titi, Jelebu, Negeri Sembilan, Malaysia were used as a starting material. The shoots were cut from rhizome and were used as explants for micropropagation. The shoots were excised at 1.5 cm from runners and washed under running tap water for 1 h. The shoots were then agitated and soaked in warm soapy water (added 2-3 drops of Tween-20) for 1 min. Then the shoots were rinse again under tap water for 15 min.

Under aseptic conditions (in laminar flow cabinet), the shoots were surface sterilized with 30% chlorox for 30 s and rinsed three times in sterile distilled water. The shoots were then blot-dried on sterile filter paper and cut into appropriate size on sterile petri dish. The sterile explants were cut into small pieces about 1.0 cm length each.

The clean explants were then cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3% (30g/L) sucrose with 20 µl BA (N6-benzyladenine) and 0.5µ l NAA (1-Napthalene Acetic Acid). The media pH was adjusted to 5.8 with 1N NaOH plus to the adding 2 g of Phytagel as the gelling agent.

The cultures were maintained in the growth room at 25 ± 2°C with 60-70% relative humidity in a 16 hour light cycle with fluorescence light of 1,000-3,000 lux. The plants were subcultured every 60 days until sufficient numbers of regenerants were available for mutagenesis. For this study, more than 2,000 regenerants were used.

Gamma irradiation

LD_{50} experiment

Gamma irradiation was carried out using ^{60}Co source (Gamma Cell 220, Physic Department, Faculty of Science, University Malaya, Kuala Lumpur, Malaysia). Regenerants were excised and transferred to 30 ml universal container with growth regulator-free semi-solid MS medium. One universal container consists of ten regenerants. The regenerants were cultured for 7 days in the medium before being subjected to mutagenesis experiment.

LD_{50} experiment was done for optimizing the suitable dose to induce mutation. For the LD_{50} experiment, regenerants were exposed to different doses 0-800 Gray (Gy). After one-day treatment, the irradiated plants were transferred into fresh MS medium with 20µl BA and 0.5µl NAA individually in 30 ml universal container. Observations to access plant survival following treatment with different high dosages of gamma ray were made after 30, 40 and 60 days in culture; however these subjected to high dosages were only occurred after 60 days.

Mutagenesis experiment

For the mutagenesis experiment, optimum dosage of the LD_{50} that was 25 Gy was used as the treatment dosage. More than 2,000 regenerants of *C. willisii* were exposed to acute irradiation of ^{60}Co at treatment dosage. After one-day following treatment, the irradiated individual shoots were transferred into fresh MS medium supplemented with 20µl BA and 0.5µl NAA in 30 ml universal container each.

After 60 days in culture, new shoots were seen had developed. The 0 Gy plants served as the control plants. A few variants were observed from irradiated plants in some of the containers, some irradiated plants produce normal shoots, but some did not survive. Shoots from control and selected variant were subcultured onto fresh medium. This is now the M_1 generation. The variant shoots were selected again and subcultured repeatedly until the 4th generation (M_4) to ensure stability of mutants. Control plants were also subcultured to the M_4 generation.

DNA extraction and Inter-Retrotransposon Amplified Polymorphism (IRAP) analysis

In most of the plant genomes, retrotransposon are highly abundant and dispersed. The characters of ubiquitous distribution, high copy number and widespread chromosomal dispersion of the retrotransposon have the potential to be used as DNA-based marker system (Kalendar *et al.*, 1999; Kalendar, 2005; Teo, 2005). The IRAP markers were successfully used in phylogenetic analysis such as in *Spartina* sp. (Baumel, 2002), banana (Teo, 2005), *Brassica* sp. (Karine, 2004) and apple (Kristiina, 2006).

Genomic DNA of D1 mutant, G1 mutant and Control (C) of the Cryptocorynes was extracted from young fresh leaves as previously described in Doyle and Doyle (1990). Following extraction, genomic DNA samples were diluted with sterile distilled water to 50 ng/l. The IRAP-PCR was performed in a 20µl reaction mixture (rxn) containing 2µl DNA samples, 1µl (5 pmol) primer A and 1µl (5 pmol) primer B, 16µl sterile distilled water and Maxime PCR Premix (i-Taq; for 20 l rxn). Component in 20 µl reaction Premix: 2.5 U *i*-Taq^{TM} DNA Polymerase, 2.5 mM each dNTPs, 1X reaction buffer and 1X gel loading buffer).

Amplification was performed using Biometra, T. personal Thermocycler. The PCR reaction parameters consists of: 95°C, 2 min; 40 cycles of 95°C, 1 min; annealing at 36.3°C, 1 min; 72°C, a final extension at 72°C, 10 min. PCR products were analyzed by electrophoresis on 1% (w/v) agarose gel and detected by ethidium bromide staining. The IRAP banding pattern was scored using Gel-Pro Imager (The Integrated Solution).

Table 3: Primers for IRAP

Name	Retrotransposon source	Sequence	Accession, position
LTR6149	BARE-1→	CTCGCTCGCCCACTACATCAACCGCGT TTATT	Z17327 1993-2012
LTR6150	BARE-1←	CTGGTTCGGCCCATGTCTATGTATCCACACATGTA	Z1 7327 418-439
5'LTR1	BARE-1←	TTGCCTCTAGGGCATATTTCCAACA	Z17327 1-26
5'LTR2	BARE-1←	ATCATTCCCTCTAGGGCATAATTC	Z17327 314-338
3'LTR	BARE-1→	TGTTTCCCATGCGACGTTCCCCAACA	Z17327 2112-2138
Sukkula	*Sukkula* →	GATAGGGTCGCATCTTGGGCGTGAC	Ay054376 4301-4326
Nikita	*Nikita* →	CGCATTTGTTCAAGCCTAAACC	Ay078073 Ay078074 Ay078075 1-22

Results and Discussion

LD_{50} experiment

The results of gamma irradiation for the LD_{50} experiments are shown in Fig. 1 and Fig. 2. At high dosage experiments (600, 700 and 800 Gy), more than 50% of the irradiated plants survived after 30 days of incubation. After 40 days incubation, the 0, 100, 150, and 200 Gy irradiated explants still showed more than 50% survival rates. The 250 and 300 Gy irradiated explants showed less than 50% survival rates. All explants unfortunately died when exposed to dosage higher than 400 Gy.

After 60 days incubation, all explants died when exposed to gamma irradiation at or more than 100 Gy. The results showing that the LD_{50} can only be achieved if the explants were exposed to dosage lower than 100 Gy.

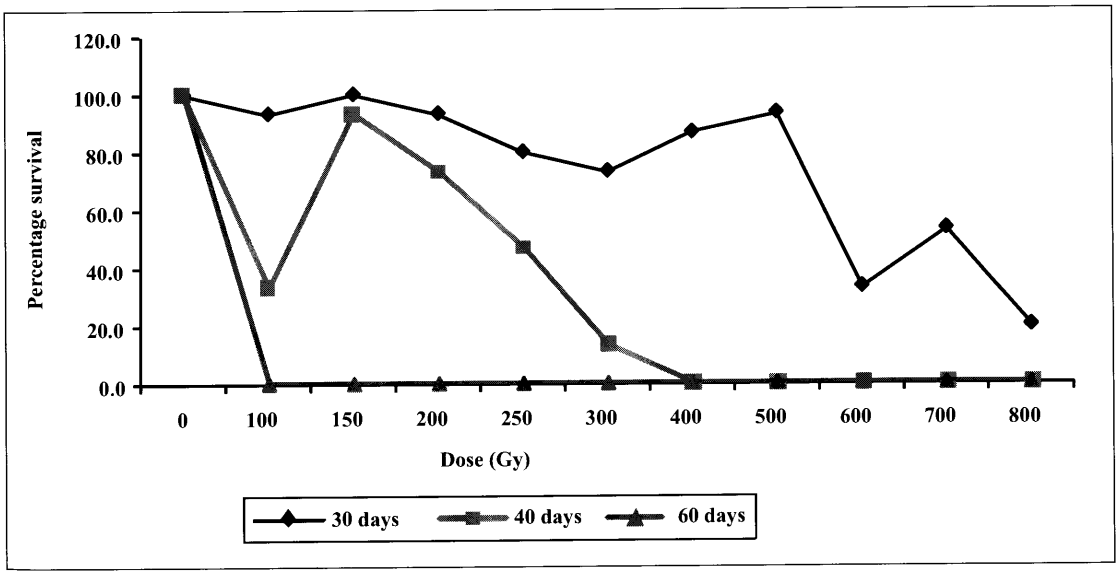


Figure 1: Percentage of survival of *Cryptocoryne willisii* on different high dosages of gamma irradiation after 30, 40 and 60 days incubations

At lower dosage experiment (Fig. 2), the 40 to 100 Gy irradiated plants were totally dead after 60 days of irradiation. From the experiment, the LD_{50} is between 0 to 35 Gy. And from best linear graph (Fig. 3), the LD_{50} is 25 Gy.

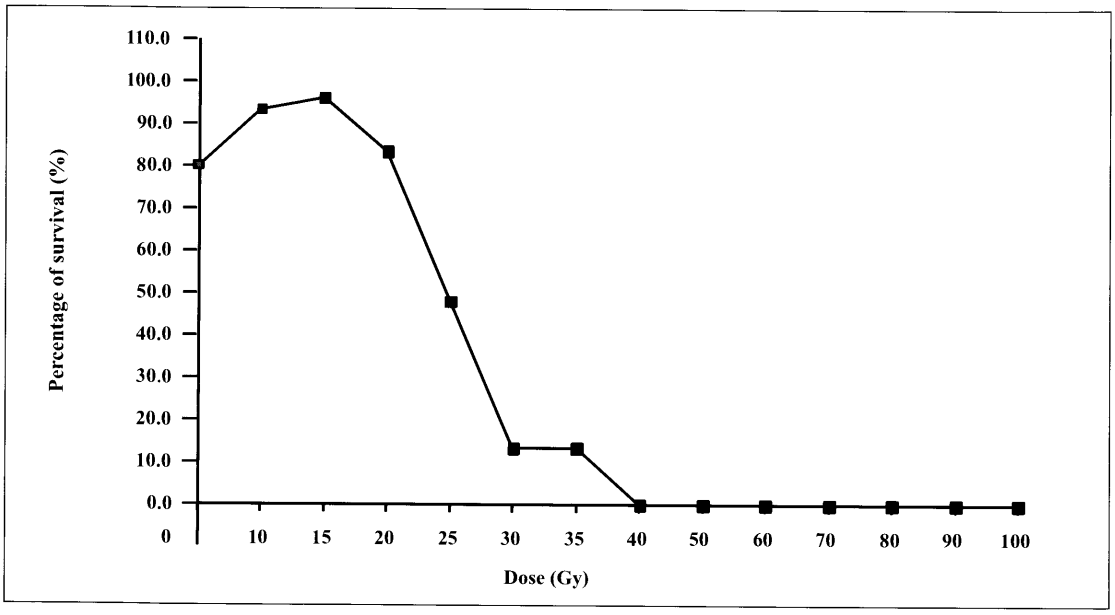


Figure 2: Percentage of survival of *C. willisii* on different low dosages of gamma irradiation after 60 days incubations

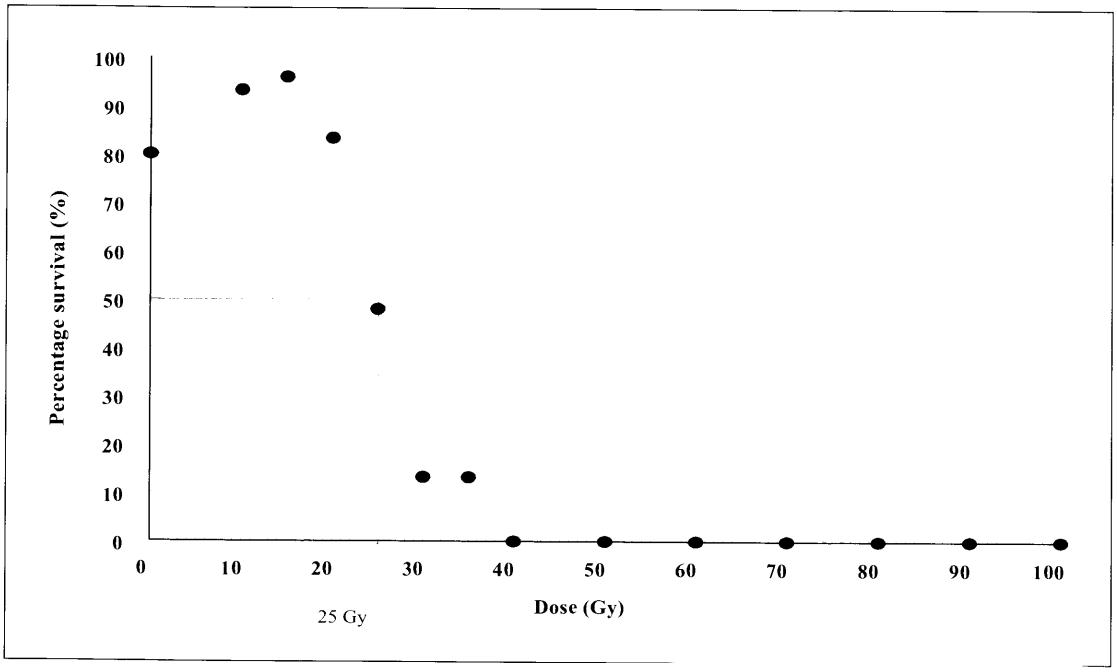


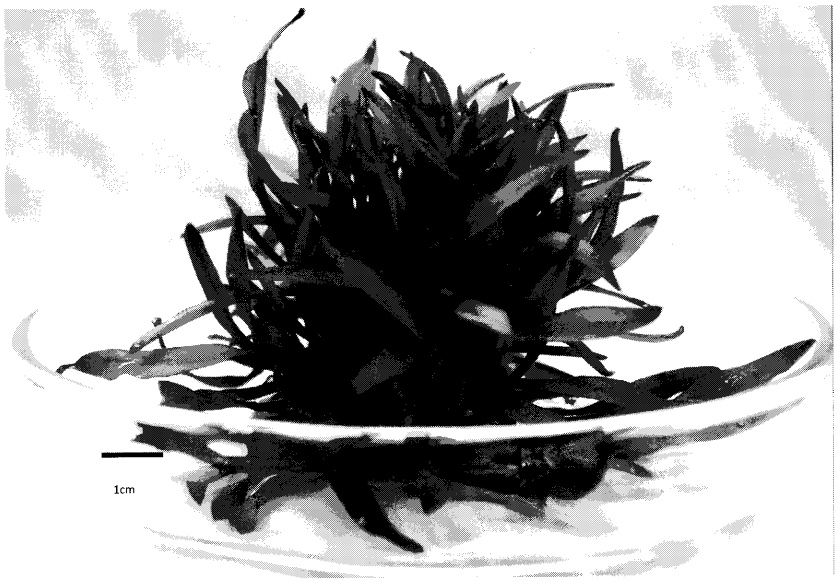
Figure 3: LD_{50} for *C. willisii* after 60 days irradiation at different low dose

Mutagenesis experiment

The shoots were subcultured repeatedly until the 4th generation (M_4) to ensure stability of the mutants. Although initially many variants were produced, only two mutants remain morphologically stable. The significant value of the F test in the ANOVA table is 0.000. Reject the hypothesis that means length of the plants are equal across different groups.

The means lengths of the plants are significantly different between the groups. Mean length of normal plants (Picture 1) is 5.18 ± 1.57 cm, mean length for D1 plants (Picture 2) is 3.53 ± 0.89 cm and mean length for G1 plants (Picture 3) is 6.92 ± 1.79 cm.

Picture 1 : Normal plants



Picture 2: Mutant D1, plants showing dwarfness compare to control plants

Picture 3: Mutant G1, plants colours are brownish and taller than control plants

IRAP analysis

A total of 36 combinations of primers were screened for marker of the mutant in the present experiment. Out of these combinations, two of the primers were sensitive enough to distinguished the D1 mutant and the G1 mutant from control plants. The analysis revealed two polymorphic bands (225 bp and 320 bp) using Nikita primer for the D1 mutant (Fig. 4) and revealed two polymorphic bands (240 bp and 300 bp) using combination of 3'LTR primer and LTR 6149 primer for the G1 mutant (Fig. 5). G1 mutant can only be distinguished when the combination primers of 3'LTR primer and LTR 6149 were used.

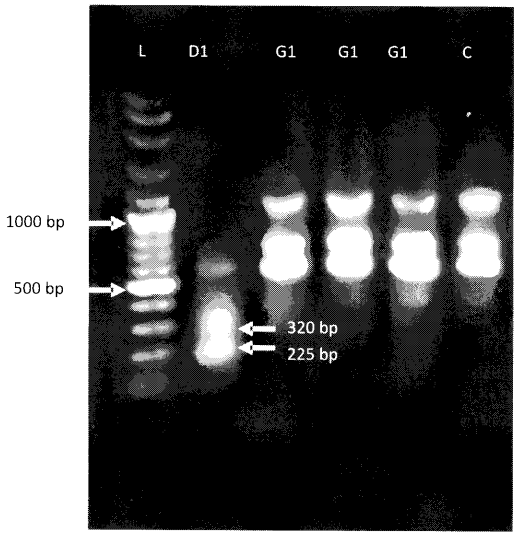


Figure 4: IRAP profile of the D1 mutant, G1 mutants and C (control) plant of C. *willisii* obtained with primer Nikita showing two polymorphic bands (arrows). L: 100 bp plus DNA ladder

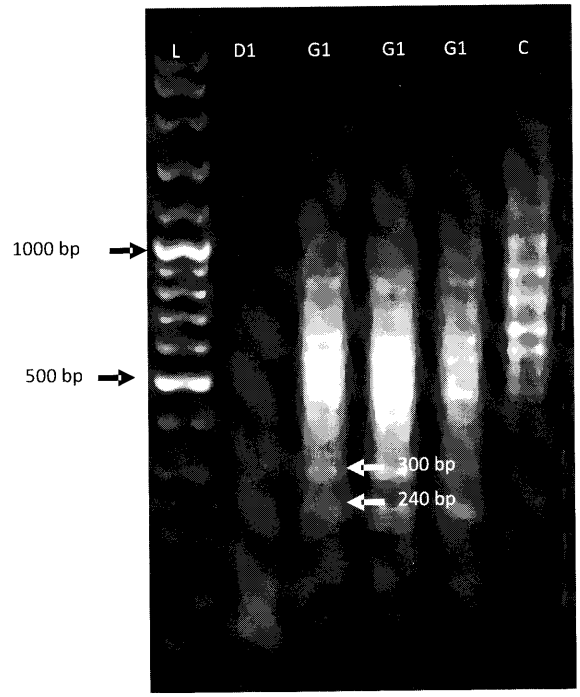


Figure 5: IRAP profile of the D1 mutant, G1 mutants and C (control) plant of *C. willisii* obtained with primer 3'LTR x LTR6149 showing two polymorphic bands (arrows). L: 100 bp plus DNA ladder

Among the main advantages of *in vitro* mutagenesis is the ability to uniformly treat large number of cells with mutagen and the cells are able to grow in uniformly under control environment. Multiplication of the mutant clones is possible via micropropagation.

In this study, all regenerants were showing necrosis when exposed to high dosage of gamma irradiation. Only when the regenerant were subject to lower dose of the gamma ray, the LD_{50} can be achieved. The LD_{50} in the study is 25 Gy and the treatments for mutagenesis are between 0 to 30 Gy. After treatment with gamma irradiation, some of the new shoots showed differences in their morphology, where some showed necrosis and others did not show any changes. These morphologically different progenies are often called variants.

In the present study, many variants were established after irradiation, such as variants with curly leaves, pink petiole, albino plants, dwarf plants and taller plants were developed at M_1 generation. These variants were selected and subcultured until M_4 generation to confirm stability.

All the variants were reverted to normal plants (control) after the second subculture (M_2) except for variants with two characters, the dwarf and the taller plants. At this M_4 generation, the two variants were stable and were called mutants. The first mutant, D1 is smaller in sizes compare to normal plants (C), particularly its leaf size and light green leaves. The second mutant, G1 had bigger and larger leaves compared to normal plants (C), with brownish leaf colour.

Beside the differences in morphology of the mutant plants, conformations of the mutants were also done using molecular markers. In this study, the IRAP markers were used to distinguish the D1 and G1 genomes from normal *C. willisii* genomes. The analysis revealed two polymorphic bands (225 bp and 320 bp) using Nikita primer for the D1 mutant and two polymorphic bands (240 bp and 300 bp) using

combination of 3'LTR primer and LTR 6149 primer for the G1 mutant. From this study, it is clear that *in vitro* mutagenesis proves to be a very promising way to produce new variety for *C. willisii* and other aquatic plants.

Acknowledgement

The authors would like to acknowledge Plant Biotechnology Incubator Unit, Institute of Science, Faculty of Science, University Malaya and Freshwater Fisheries Research Division, FRI Glami Lemi, Department of Fisheries for their providing the facilities and financial support of the project.

References

Baumel, A., Ainouche, M., Kalendar, R. and Schulman, A.H. 2002. Inter-Retrotransposon Amplified Polymorphsm (IRAP) and Retrotrasposon Microsatellite Amplified Polymorphism (REMAP) in population of the young allopolyploid species *Spartina angelica Hubbard* (Poaceae). *Molecular Biology Evolution*, **19**: 1218-1227

Cook, Christopher, D.K. 1996. Aquatic and Wetland Plants of India. Oxford University Press Inc. New York.

Dariusz, G. 2006. Transposon insertion polymorphismas a new source of molecular markers. *Journal of Fruit and Ornamental Plant Research*, **14** (Suppl. 1): 21-29.

De Wit, H. C. D. 1964. Aquarium Plants. London Blandford Press Ltd.

Doyle, J.J. and Doyle, J.L. 1990. A rapid total DNA preparation procedure for fresh plant tissue. *Focus*, **12**: 13-15

Hiscock, P. 2005. Mini Encyclopedia. Aquarium Plant: Comprehensive coverage, from growing them to perfection to choosing the best varieties. Barron's Educational Series, Inc.

Kalendar, R., Grab, T., Regina M., Suoniemi, A. and Schulman, A.H. 1999. IRAP and REMAP: Two new retrotransposon-based DNA fingerprinting techniques. *Theoretical and Applied Genetics*, **98:**704-711.

Karine, A. and J. S. (Pat) Heslop-Harrison 2004. The diversity of retroelements in diploid and allotetraploid Brassica species. *Plant Molecular Biology*, **54:** 895–909.

Kristiina Antonius-Klemola, Ruslan, K. and Alan, H. S. 2006. TRIM retrotransposons occur in apple and are polymorphic between varieties but not sports. *Theoretical and Applied Genetics*, **16**: 23-30.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiology Plant*, **15**: 431-497.

Rataj, K. and Horeman, T.J. 1977. Aquarium Plants: their identification, cultivation and ecology. T.F.H. Publications, Inc. Ltd.

Teo, C.H., Tan, S.H., Ho, C.L., Qamaruz, F.Z., Othman, R.Y., Heslop-Harrison, J.S., Kalendar R. and Schulman, A.H. 2005. Genome constitution and classification using retrotransposon-based markers in the orphan crop banana. *Journal of Plant Biology*, **48(1):**96-105.

Windelov, H. 1987. Aquarium Plants. T.F.H. Neptune City, N.J.