INDUCED MUTATION OF *IN VITRO* AQUATIC PLANT, *CRYPTOCORYNE XWILLISII* REITZ BY USING GAMMA IRRDIATION AND IRAP ANALYSES TO DISTINGUISH THE SPORTS (CLONAL MUTATION)

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ABSTRACT

One part of ornamental fish industry is the aquatic plants. Ornamental fish industry is identifying as one of National Key Economic Area (NKEA) for Malaysia, under business opportunity. *Cryptocoryne* x*willisii* Engler ex Baum is one of the highly demanded aquatic plants in international market. Unfortunately, the plants take months to grow, seldom flowering and no viable seeds. The studies were done to mass-produce the plants by tissue culture and to develop new variety by mutation.

Tissue culture of water trumpet, *Cryptocoryne* x*willisii* Engler ex Baum, was developed using Murashige and Skoog 1962 (MS) medium, which contained two different plant growth regulators, namely 6-benzyladenine purine (BAP) and α-naphthalene acetic acid (NAA). Seven different concentrations of BAP (0, 0.5, 1.0, 5.0, 10.0, 20.0 and 40.0 μM) and NAA (0, 0.5, 1.0, 5.0, 10.0, 20.0 and 40.0 μM) were investigated using a two-factor factorial design with 10 replicates. Results of the experiments were collected and analysed after 40 days of culture.

The results showed that the effects of plant growth regulators on increasing the number of shoots were highly significant ($p<0.01$). The MS medium containing 1.0 μM BAP alone was the optimum concentration producing 6.8 ± 1.75 shoots per explant. This was the minimum formula concentration of BAP used that produced the highest number of shoots. Results showed that there were twelve hormone combinations giving high mean values of between 4 to 6 shoots per explant. All of the explants cultured in these media produced shoot (100 %).

Although all the explants gave a positive response in terms of regeneration, they however differed in the number and size of shoots produced. Analysis of the data using

ANOVA indicated that the number of shoots produced was significantly influenced by both BAP and NAA concentrations simultaneously. This was suggested by the significance of the interactions between BAP and NAA which showed that BAP concentrations affected the number of shoots differently for each concentrations of NAA tested and vice versa.

Two new varieties of *C.* x*willisii* have been developed through mutagenesis in this work. Shoot tips explants of *C.* x*willisii* were subjected to a range of ^{60}Co gamma ray irradiation of 0, 100, 200, 300, 400, 500, 600, 700 and 800 Gray. Results from experiments showed that the LD_{50} for the tissue culture plants of *C.* x*willisii* was at 32 Gy dose. And was therefore, less than 32 Gray was used as the appropriate dosage for induced mutations of the plants.

About two thousand of the shoot tips explants were irradiated using the 25 Gy and variants from the M_1, M_2, M_3 and M_4 generations were screened for morphological differences. The variants 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 were shown to remain stable. The mutants obtained were dwarf plants (D1) and plants of taller stature with pigmented leaves (G1) than the controls. This was verified from the significant value of the F test in the ANOVA where $P<0.05$.

The Inter-Retrotransposon Amplified Polymorphism (IRAP) markers distinguished the D1 and G1 genomes from normal *C. xwillisii* 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.

ABSTRAK

Tumbuhan akuatik merupakan salah satu bahagian dalam industri ikan hiasan. Industri ikan hiasan telah dikenalpasti sebagai salah satu bidang keutamaan negara (National Key Economic Area (NKEA)), dibawah peluang perniagaan (business opportunity). *Cryptocoryne* x*willisii* Engler ex Baum merupakan salah satu tumbuhan akuatik yang mendapat permintaan yang tinggi dalam pasaran antarabangsa. Akan tetapi, tumbuhan ini mengambil masa yang lama untuk membesar, jarang berbunga dan tiada biji benih yang viable. Kajian yang dijalankan adalah untuk menghasilkan tumbuhan ini secara pukal melalui kaedah kultur tisu dan penghasilan variati baru melalui kaedah mutasi.

Tisu kultur trumpet air, *Cryptocoryne* x*willisii* Engler ex Baum, telah dijalankan menggunakan medium Murashige dan Skoog 1962 (MS) yang mengandungi dua pengalak pertumbuhan pokok yang berlainan, iaitu 6-benziladenin purin (BAP) dan α-naftalina asetik asid (NAA). Tujuh kepekatan yang berlainan BAP (0, 0.5, 1.0, 5.0, 10.0, 20.0 and 40.0 μM) dan NAA (0, 0.5, 1.0, 5.0, 10.0, 20.0 and 40.0 μM) telah dikaji menggunakan kaedah dua faktor faktorial dengan sepuluh replikasi. Keputusan kajian diambil dan dianalisa selepas 40 hari dikultur.

Keputusan menunjukkan bahawa kesan pengalak tumbesaran tumbuhan untuk meningkatkan bilangan pucuk adalah sangat signifikan ($p<0.01$). Medium MS yang mengandungi 1.0 μM BAP sahaja adalah kepekatan yang optima yang menghasilkan 6.8 ± 1.75 pucuk bagi setiap eksplan. Ini adalah formula yang minima yang boleh meningkatkan bilangan pucuk. Keputusan kajian menunjukkan terdapat dua belas kombinasi hormon yang memberi nilai purata diantara 4 hingga 6 pucuk setiap eksplan. Kesemua eksplan yang dikultur di atas media menghasilkan pucuk (100%).

Walaupun, kesemua eksplan memberi tindakbalas yang positif dari segi pertumbuhan namun keputusannya berbeza dari segi bilangan dan saiz pucuk yang dihasilkan. Analisa data dibuat menggunakan ANOVA menunjukkan bilangan pucuk yang terhasil dipengaruhi oleh BAP dan NAA dengan serentak. Ini dicadangkan berdasarkan signifikan interaksi antara BAP dan NAA di mana ia menunjukkan kepekatan BAP memberi kesan yang berbeza ke atas bilangan pucuk pada setiap aras kepekatan NAA dan juga sebaliknya.

Dua variati baru *C.* x*willisii* telah berjaya dibangunkan dalam kajian ini melalui kaedah mutagenesis. Pucuk eksplan *C.* x*willisii* telah didedahkan kepada satu julat penyinaran Sinaran Gamma ^{60}Co iaitu 0, 100, 200, 300, 400, 500, 600, 700 dan 800 Gray. Keputusan kajian menunjukkan dos kematian 50% (LD50) untuk tumbuhan tisu kultur *Cryptocoryne* x*willisii* ialah pada dos 32 Gray. Dan oleh itu, nilai dos yang lebih rendah dari dos tersebut telah digunakan untuk mengakibatkan mutasi pada tumbuhan.

Sebanyak dua ribu pucuk eksplan telah diradiasi menggunakan dos 25 Gray dan varian-varian dari generasi M_1, M_2, M_3 dan M_4 telah dipilih untuk perubahan morfologi. Pucuk-pucuk varian telah disubkultur berulang kali sehingga generasi ke 4 (M_4), ini bagi memastikan kestabilan mutan tersebut. Walaupun banyak regeneran dengan pelbagai sifat morfologi yang berbeza dihasilkan tetapi hanya dua sahaja mutan yang stabil diperolehi. Mutan yang diperolehi ialah tumbuhan kerdil (D1) dan tumbuhan yang lebih tinggi dari tumbuhan kawalan. Ini telah diverifikasi dari nilai signifikan ujian F dalam ANOVA dimana $P<0.05$.

Penanda Inter-Retrotransposon Amplified Polymorphism (IRAP) dapat membezakan genome D1 dan G1 dari genome *C.* x*willisii* normal. Analisis yang dijalankan mendapati untuk mutan D1, didapati ada dua jalur khusus iaitu jalur 325 bp dan

jalur 420 bp dengan menggunakan primer Nikita manakala untuk mutan G1, didapati ada dua jalur khusus iaitu jalur 240 bp dan jalur 300 bp dengan menggunakan kombinasi primer 3'LTR dan primer LTR 6149.

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Dedicated to:

My loving husband M.Husni Sulor,
and my wonderful children Aisyah, Izzah, Izzati, Firdaus and Safiyya,
My both parents and parents in law,
And my relatives.

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ABBREVIATIONS AND ACRONYMS

%	-	Percent
^{60}Co	-	Cobalt 60
AFLP	-	Amplified fragment length polymorphism
BAP	-	N6-benzyladenine Purine
NAA	-	1-napthaleneacetic acid
bp	-	base pair
CAPs	-	cleaved amplified polymorphism sequences
CTAB	-	hexadecyltrimethylammonium bromide
DF	-	Dilution Factor
DNA	-	Deoxyribonucleic Acid
DOF	-	Department of Fisheries
EDTA	-	Ethylenediaminetetraacetic Acid
ELF	-	extremely low frequency
EtBr	-	Etidium Bromide
FRI	-	Fisheries Research Institute
g/L	-	gram per liter
GDP	-	Gross Domestic Product
Gy	-	Gray
IRAP	-	Inter-retrotransposon amplified polymorphism
kPa	-	kilopascal
LD_{50}	-	Lethal Dose 50%
LS	-	Linsmaier and Skoog
MGI	-	Malaysia Gross Income
min	-	minute
mM	-	milimolar
MS	-	Murashige and Skoog medium
MSO	-	Murashige and Skoog medium without hormone
NaCl	-	Sodium chloride
NaOCl	-	Sodium Hypo chloride
NaOH	-	Sodium hydroxides
NH_4 acetate	-	Ammonium acetate
ºC	-	Degree Celsius
PCR	-	Polymerase Chain Reaction
pmol	-	Pico mol
RAMP	-	randomly amplified microsatellite polymorphisms
RAPD	-	Random amplification of polymorphic DNA
RBIP	-	Retrotransposon-based insertion polymorphism
REMAP	-	REtrotransposon-microsatellite amplified polymorphism
RFLP	-	Restriction fragment length polymorphism
RNAse	-	Ribonuclease
rpm	-	round per minute
rxn	-	reaction mixture
S-SAP	-	sequence-specific amplification polymorphism
SCAR	-	sequence characterized amplified regions
SNPs	-	single nucleotide polymorphism

SRAP	-	sequence-related amplified polymorphism
SSCP	-	single strand conformation polymorphism
TRAP	-	target region amplification polymorphism
Tris HCL	-	Tris Hydrochloric
USD	-	United States Dollar
UV	-	Ultra violet
v/v	-	volume per volume
VLF	-	very low frequency
w/v	-	weight per volume
μg	-	microgram
μl	-	micro liter
μM	-	micro molar

CHAPTER 1 INTRODUCTION

CHAPTER 2 LITERATURE REVIEW

CHAPTER 3 MATERIALS AND METHODS

CHAPTER 4 RESULTS

CHAPTER 5 DISCUSSION