3.0 MATERIALS AND METHODS

3.1 Plants Materials

Samples of *C.* x*willisii,* used as starting materials, were received from Fisheries Research Institute Glami Lemi (FRI Glami Lemi), Freshwater Fisheries Research Division, Department of Fisheries Malaysia, Titi, 71650 Jelebu, N.Sembilan, Malaysia. The shoots were cut from rhizomes of the plants and were used as explants for tissue culture.

3.1.1 Surface Sterilization Of Plants Materials

Shoots sizes around 1.5 cm were excised from runners and washed under running tap water for 1 hour. The shoots were then stirred and soaked in warm soapy water (added 2-3 drops of biological detergent (tween 20)) for one minute. Then the shoots were rinsed again under tap water for 15 minutes.

Under aseptic conditions (in laminar flow cabinet), the surfaces of the shoots were surface sterilized with 30% (v/v) chlorox (active ingredient 5.25% sodium hypochlorite) for 30 second and rinsed three times in sterile distilled water. The shoots were then dried on sterile filter paper.

3.2 Preparation Of Culture Media

For media preparation, the following protocol was carried out. The Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) were prepared using formula as

described in Appendix Table 3.2.1. MS medium was prepared by using stock solution of basic MS Salt, 0.1 g myo-inositol and 3% (w/v) sucrose. The medium was adjusted to pH 5.8 with 1N NaOH and 2 g of Phytagel (Sigma) without any hormone. MS medium without hormone is labelled MSO.

The medium was stirred until all chemicals were dissolved. Then the medium heated in the microwave for 13 minutes (for 1 liter medium) until boiling. The media was left for cooling before being dispensed into jam jar bottles. From 1 liter of medium, about 30 ml of medium was dispensed into each 150 ml glass jars and closed with polypropylene lids. The prepared culture media jars were autoclaved at 121°C at 118 kPa for 20 min.

For media optimization study, MS media were prepared and supplemented with various concentrations of 6-benzyladenine (BA) and α-naphthalene acetic acid (NAA) of 0 μM, 0.5 μM, 1.0 μM, 5.0 μM, 10.0 μM, 20.0 μM and 40.0 μM respectively (Appendix Table 3.2.2). All media were adjusted to pH 5.8 with 1N NaOH and 2 g of Phytagel (Sigma) were added into the media. The media were microwave for 13 minutes. The 30 ml media were dispensed into 150 ml glass jars and sealed with polypropylene lids. Then the glass jars were also autoclaved at 118 kPa for 20 min at 121°C.

3.3 Establishment Of Shoot Tips Culture

The sterile and dried explants were dissected on sterile petri dish leaving only the inner part of the shoots (sizes of about 1.0 cm). The sterile explants were then cultured on Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) supplemented with 3% (30g/L) sucrose with 20μl BA (N6-benzyladenine) and 0.5μl

NAA (1-napthaleneacetic acid) hormone. 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, 30 μmolm^{-2}s^{-1}. The *in vitro* shoot tips cultures were then established as the source of explants for tissue culture studies and induced mutation experiment. The plants were grown for 60 days and sub cultured until sufficient number of regenerants.

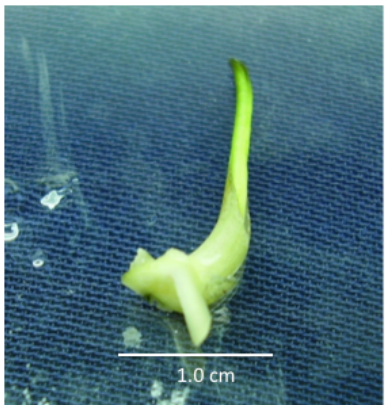

Plate 3.3.1 Excised shoot explant (about 1.0 cm) for shoot tip cultures initiation.

3.3.1 Media Optimization With Different PGR Combination

For media optimization study, the established *in vitro* shoot tips cultures were used as the source of explants. The shoot tips were cultured on MS media supplemented with various concentrations of 6-benzyladenine (BA) and α-naphthalene acetic acid (NAA) of 0 μM, 0.5 μM, 1.0 μM, 5.0 μM, 10.0 μM, 20.0 μM and 40.0 μM respectively. Ten samples were cultured for each combination of hormone. Explants were incubated in growth room at 25 ± 2°C with a 16-h photoperiod (cool white fluorescent light, 30 μmolm^{-2}s^{-1}). After forty days, data on number of shoots were observed and counted.

3.4 Mutagenesis Induction

3.4.1 Determination Of Lethal Dose LD_{50} *(Gamma Irradiation)*

The *in vitro* shoot tips cultures were also established as the source of explants for mutation induction studies. For these studies, gamma irradiation was chosen due to its acute effect to the explants.

Gamma irradiation was carried out using ^{60}Co source (Gammacell 220) at Physic Department, Faculty of Science, University Malaya, Kuala Lumpur, Malaysia. The *in vitro* shoot tips cultures (regenerants) were excised and transferred to 30 ml universal containers with solidified MS media without growth regulator. One universal container consists of ten regenerants. The regenerants were cultured for 7 days in the media, before being sent for gamma irradiation.

For the mutagenesis experiment, LD_{50} (Lethal Dose) was initially carried out on the *in vitro* shoot tips cultures to optimize a suitable dose for induce mutation. For the LD_{50} experiment, regenerants were exposed to different high dosages (0-800 Gray; Gy): 0, 100, 150, 200, 250, 300, 400, 500, 600, 700 and 800 Gy of gamma ray. The regenerants were also subjected to different low dosages (0-100 Gy): 0, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90 and 100 Gy of gamma ray.

After one-day treatment, the irradiated plants were transferred into new MS media with 2.0 mg/l BA and 0.5 mg/l NAA hormone individually in 30 ml universal containers.

Observation was done on the plant survival after subjected to different high dosage of gamma ray was made after 30, 40 and 60 days culture. Only after 60 days did the plants explants exhibited any results. And the observations on the plant survival

after subjected to different low dosage of gamma ray were also made only after 60 days culture.

3.4.2 Dose Rate Calculation For ^{60}Co unit (Gammacell 220).

For 1 month = 30 x 24 x 60 x 60 = 2592000 seconds.

The dose rate after one month $Dt = Do\ e^{-\lambda t}$, where λ is the decay constant and = 4.1681 x $10^{-9}s^{-1}$.

Do is the initial dose rate for Gammacell 220 which equal = 0.525 Gy/sec (Nov. 1995).

$Dt = 0.525e^{-4.1681 \times 10\text{-}9} \times 2592000 = 0.519$ Gy/sec.

Calculation for Gray

Example. Dose rate for May 2007 = 0.11794 Gy/sec

0.11794 Gray – 1 second

$$10 \text{ Gray} = \frac{10 \text{ Gray} \times 1 \text{ second}}{0.11794 \text{ Gray}} = 84.789 \text{ second}$$

For 10 Gray exposures, the plants were exposed to the gamma irradiation for the duration of 85 seconds. In each different month, the time of exposure would be different because the dose rates were different.

3.4.3 Gamma Irradiation Experiment

For the mutagenesis experiment, optimum dosage (25 Gy) of the LD_{50} plus 0, 10, 15, 20 and 30 Gy were used as the treatment dosages. More than 2000 regenerants of *C.* x*willisii* were exposed to acute irradiation of ^{60}*Co* at treatment dosages. After one

day treatment, the irradiated plants were also transferred into new MS medium with 20µl BA and 0.5µl NAA hormone individually in 30 ml universal containers.

After 60 days culture, new shoots were produced. The 0 Gy plants served as the control plants. A few variants could be seen from irradiated plants in some container, some irradiated plant produced normal shoots and some plant did not survived. Shoots from the control and selected variants were subcultured in new MS medium with the same hormone. This was labeled as the M_1 generation. The variants shoots were selected again and subcultured repeatedly until the 4^{th} generation (M_4) to ensure stability of mutants. Control plants were also subcultured until M_4.

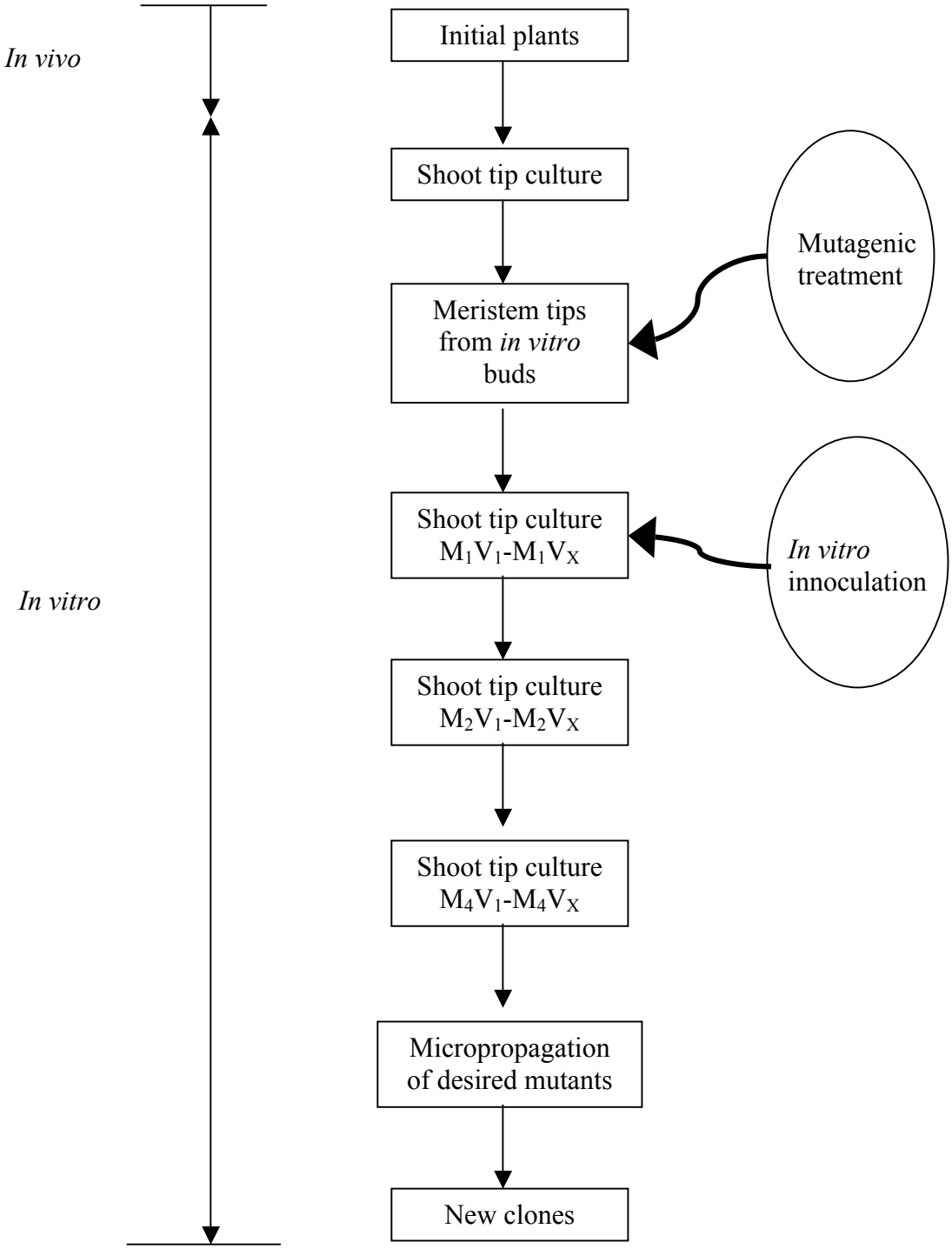

Figure 3.4.3.1. *In vitro* induced mutation for *C.* x*willisii* adapted from Basiran *et al,* (1998).

3.5 Phylogenetic Studies Using Inter-Retrotransposon Amplified Polymorphism (IRAP)

3.5.1 Plants Materials

Twenty varieties and species of Cryptocoryne from the Malaysia and Sri Lanka were used (Appendix 3.5.1). The Malaysian samples comprised eight samples from Sarawak, four samples from Johor, and two samples from Pahang. The samples collection was done by FRI Glami Lemi from the various locations. And another six samples (origin from Sri Lanka) were obtained from aquatic plants farm in Johor. All samples were taken from the germplasm collections of the Freshwater Fisheries Institute (FRI Glami Lemi), Department of Fisheries Malaysia in Titi, Jelebu, Negeri Sembilan.

3.5.2 DNA Extraction

Genomic DNA of the Cryptocoryne was extracted from one gram of the young leaves as previously described in Doyle and Doyle (1990).

DNA was extracted using a modified Doyle and Doyle CTAB methods. 5-7.5 ml of CTAB isolation buffer (2% (w/v) hexadecyltrimethylammonium bromide [CTAB: Sigma H-5882], 1.4M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA (Ethylenediaminetetraacetic Acid), 100 mM Tris-HCl pH 8) was preheated in a polypropylene tube to 60°C in water bath. Liquid nitrogen was used to grind 1.0 g fresh leaf tissue by using the cold mortar.

The ground sample was added into the CTAB isolation buffer and incubated at 60°C for 30-60 min with optional occasional gentle swirling. Then extraction was done using 5ml of chloroform-isoamyl alcohol (24:1), and mixed gently but thoroughly for 10 min. Then the sample was spun down by centrifuged at 5000 rpm, 25°C for 10 min. The clear aqueous (supernatant) was removed using wide bore pipet and transferred to clean polypropylene tube and extracted again using fresh chloroform-isoamyl alcohol (5ml). Then the sample was spun down by centrifuged at 5000 rpm was spun again in centrifuge at 5000 rpm, 25°C for 10 min. The clear aqueous (supernatant) was removed using wide bore pipet and transferred to a clean polypropylene tube.

50 µl RNAse A (100 µg/ml [0.01g RNAse, 10µl (10mM) Tris HCl pH 7.5, 6µl (15mM) NaCl, 984µl ultra pure water]) was added into the supernatant, mixed and incubated at room temperature for 30 min. Then 0.6 volumes (3ml) ice cold isopropanol was added, and the mixture was left overnight.

5 ml washing solution (76% (v/v) absolute ethanol, 10 mM NH_4 acetate, ultra pure water) was added to the mixture and mixed gently to precipitate nucleic acids. The mixture was spin in centrifuge at 5000 rpm, 4°C for 10 min. The pellet were then collected and 2 volumes (10 ml) supernatant was discarded; 2 volumes (10 ml) of ice cold absolute ethanol was added and the mixture were collected and kept for 1 hour at -20°C. The mixture was spun down again at 5000 rpm, 4°C for 10 min. Supernatant was discarded and 70% ethanol (1 ml) was used to wash the pellet. Centrifuged at 5000 rpm, 4°C for 10 min to spin down the pellet. Supernatant was discarded and the final pellet was drained on the filter paper. The pellet was dissolved in 100 µl cold (0°C) TE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0, ultra pure water). The DNA samples were then stored at -20° C.

3.5.3 DNA Quantification

The extracted DNA was then quantified using Eppendorf Biophotometer. The genomic was diluted using the formula below before OD reading was taken.

(OD_{260} x Dilution Factor (DF) x 50)/1000

1 OD_{260} = 50 µg/ml

DF = 100

= 990 µl TE + 10 µl DNA

All DNA samples were diluted and the OD reading for each sample was taken.

3.5.4 Gel Electrophoresis Of Genomic DNA

The quality of the DNA was determined by running the electrophoresis of the DNA to see. There should be only one single band for genomic DNA. If multiple bands were seen on the gel, the DNA could be degraded and it cannot be used for further experiment and the sample need to be re-extract again.

The first step for electrophoresis was to prepare the agarose gel. 1% (w/v) agarose was added in 1 x TBE (10 mM Tris HCl pH 8.0, 1 mM EDTA pH 8.0, and ultra-pure water) and cooked in microwave for 1 min until the agarose was dissolved. After the agarose cooled a bit, ethidium bromide (EtBr) (1 µl EtBr for every 40 ml gel) was added. The gel was poured into the plate with the comb. After the gel was

hardened, the comb was removed and the gel was mounted in the electrophoresis tank. Buffer 1 x TBE was added until it covered the gel.

18 µl DNA was mixed with 2 µl loading dye. The first lane on the gel, 10 µl (100bp) ladder was loaded. The second lane and forward, DNA mixtures were loaded. Electrophoresis was initiated at 80 V for 40 min. After 40 min, the gel was read under UV light using UV table. The bands were scored and photographed using Gel Documentation Machine (Gel-Pro Imager, The Integrated Solution).

3.5.5 IRAP Analysis On Selected Species Of Cryptocoryne

After the extraction, genomic DNA samples were diluted with sterile distilled water to 50 ng/µl. The IRAP-PCR was performed in a 20 µl reaction mixture (rxn) containing 2 µl DNA samples, 1 µl (5 pmol) primer A and 1µl (5 pmol) primer B, 16 µl sterile distilled water and Maxime PCR Premix (i-Taq; for 20 µl 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.]. The IRAP primers used as in Appendix 3.5.2 and Appendix 3.5.3 showing forty-five combinations of primers used in the study.

Amplification was performed using Biometra, T.personal Thermocycler. The PCR reaction parameters consisted of: 95°C, 2 min; 40 cycles of 95°C, 1 min; annealing at 36.3°C, 1 min; 72°C, 2 min + 3 second per cycle; 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.

3.5.6 Phylogenetic Analyses

A phylogenetic tree was constructed by a distance-based method. Evolutionary distances and phylogenetic tree was calculated by using PAUP: Phylogenetic Analysis Using Parsimony version 4.0. Results from the PCR were keyed in the table. This consisted of scores for the presence or absence of the bands of the particular mobility in accessions. Only the clear and distinguishable bands were scored. A value of '1' indicated the presence of a band of a particular mobility compared to a DNA ladder, and '0' indicated the absence of that particular band.

3.6 IRAP Analysis On Mutants

3.6.1 Plants Materials

The normal *C. xwillisii* and the two mutants, Dwarf mutant and Giant mutant were used in the study. All samples were taken from the previous induced mutation study.

3.6.2 DNA Extraction

As previously described in Section **3.5.2**

3.6.3 DNA Quantification

As previously described in Section **3.5.3**

3.6.4 IRAP Analysis On Mutant Vs Wild Type *C.xwillisii*

As previously described in Section **3.5.4.**

Analysis were done by looking for specific bands to distinguish the two mutants that were the small size mutant and bigger size mutant from normal *C. xwillisii*. Only six primers were used (Appendix Table 3.6.1), giving twenty-one primers combinations (Appendix Table 3.6.2).

3.7 Statistical Analysis For Media Optimization With Different PGR Combination

One culture bottle represents as one experimental unit and each treatment was replicated using a completely randomized design. Cultures were evaluated for shoot multiplication after forty days in culture. Main treatment effects and interactions were evaluated using Linear Models: Analysis of Variance, Factorial Design procedures developed by Statistix 8.0 Analytical Software (1985-2003) and SPSS 14.0 software for Windows Evaluation Version (SPSS Inc.).