5.0 DISCUSSION

5.1 Sterilization Technique

The materials were initially cleaned to remove any dead or superfluous tissue followed by dissecting into small pieces before washing under running tap water for one hour. This method could reduce the level of external microorganism.

The explants were soaked in warm soapy water with non-phytotoxic wetting agent Tween 20 to reduce the surface tension on the explant and thus allowed better access to sterilizing agent. Sterilizing agent used in this study is the commercial house-hold bleach Clorox which contained 5.25% (w/v) sodium hypochlorite and less toxic to plant materials. Other sterilizing agent, such as ethanol was not suitable for surface sterilization of the study explants. *C.* x*willisii* explants were very sensitive to alcohol because it caused reverse osmosis in the plant. The soft tissue of the *C.* x*willisii* could not withstand the water loss and it turned white and died.

For sterilizing other aquatic plants, sterilizing agent used usually are the same that are the ethanol and sodium hypochlorite. The differences are only on the concentration used and time applied. A few studies were done using different combinations of sterilizing agent, 50% (v/v) ethanol (1 min) and 1.05% (w/v) NaOCl (12 min) were used to dip the shoot tips of *Cryptocoryne lucens* (Kane *et al* 1990) and *Cryptocoryne wendtii* (Kane *et al* 1999) and in *Aponogeton madagascariensis*, 70% (v/v) ethanol (1 min) and 1.05% NaOCl (10min) were used to surface sterilized the inflorescence (Nathakorn 2005), for *Bacopa monnieri*, nodal segments were disinfected in 70% (v/v) ethanol (30 sec) and 25% NaOCl (25 min) (Escandón *et al* 2006) but for the shoot tips *Anubias barteri*, only 0.5% NaOCl (10 min) was used as disinfectant agent (Huang *et al* 1994).

5.2 PGR Optimization for Shoot Proliferation

The effect of culture medias and concentration of NAA on clonal micro propagation of aquatic plant, *Anubias barteri* Engler, 1979 were studied. Shoot were cultured on 4 different semi-solid medias: 1) MS media (Murashige and Skoog, 1962), 2) MS modified (half of macronutrients from normal MS), 3) LS (Linsmaier and Skoog, 1965) and 4) LS modified (half of macronutrients from normal LS). All medias supplemented with 2 mg/l N_6-Benzylaminopurine (BA) and combined with 2 different concentration of NAA: 0 mg/l and 0.25 mg/l. After 8 weeks cultured, there was no significant effect of media on shoot multiplication ($p>0.05$).

However, when considered the quality of shoot, the Anubias tissues which were cultured on media 1 and 3 gave the complete shoot which non hyperhydration explants. In addition the Anubias tissue which were culture on 4 different media combination with NAA 0 and 0.25 mg/l show significantly different on shoot and root proliferation ($p<0.05$). Maximum shoot proliferation (3.60±1.074 shoots) were observed on MS media supplement with 2 mg/l BA alone. All treatment combined with 0.25 mg/l of NAA gave the less number of shoot (means=2.55±0.749 shoots) and too many root (means=10.975±6.595 roots), number of leaf/explant and number of leaf/shoot was decreased which caused the stunt plantlet. In conclusion, the MS and LS media without NAA should be suggested for clonal micro propagation of *Anubias barteri*.

Procedures for *in vitro* establishment, axillary shoot proliferation and plantlet acclimatization of the aquatic plant, *Cryptocoryne wendtii* De Wit. were determined. Surface-sterilized rhizome shoot tips were established on a basal medium (BM) consisting of Murashige±Skoog mineral salts, 0.56 mM myo-inositol, 1.2 mM

thiamine±HCL and 87.6 mM sucrose supplemented with 2.2 mM N6-benzyladenine (BA) and 0.57 mM indole-3-acetic acid (IAA) and solidified with 0.8% TC agar. Effects of basal medium supplementation with factorial combinations of BA (0±25 mM) and IAA (0±10 mM) on axillary shoot proliferation from single-node explants were determined after 28 days. Maximum axillary shoot proliferation (sevenfold increase) occurred on medium supplemented with 20mM BA alone. Excellent microcutting rooting (100%) was achieved by direct sticking microcuttings in Metro Mix 500 soilless planting medium. Greenhouse acclimatization of rooted microcuttings was 100%. High-quality salable plants were produced within eight weeks post-transplant.

In media where both BA and NAA were used in combinations, at low concentrations of BA and increasing concentrations of NAA, the number of shoots produced were decreased accordingly, however as at low concentrations of NAA and increasing concentrations of BA, the number of shoots increased but were abnormal in appearance at concentration more than 5.0 μM. However, the highest number of shoots produced (5.4±2.0 shoots per explant) was not superior compared to media supplemented with 1μM BA alone. Abnormalities such as stunted shoots and large petioles were seen at the same PGR concentrations (10.0, 20.0, 40.0) were used.

5.2.1 Absence of BA and NAA

In this study, very low numbers of shoots were produced 2.5±1.0 shoots per explant in the absence of PGRs (BA and NAA) and a few rooted shoot tips were established. This showed that hormone auxin and cytokinin in *C.* x*willisii* were low in the plants itself and there are the needs of PGRs to increase number of shoots.

5.2.2 Absence of NAA

In MS media with absence of NAA and low concentrations of BA (0.5, 1.0, 5.0, 10.0, 20.0 μM), results also shown low number of shoots. At high concentrations of BAP (40.0 μM) even though high numbers of shoots were produced but the shoots were off types and stunted in appearance without any rooting. The stunted shoots were difficult to separate to be as individual plantlets. The dwarf effect of BA was also shown in published report by Nor Aziah *et al,* 2002. Rooted shoot tips were observed in media with 0.5 and 1.0 μM BA. The work done by Gloke *et al*, 2006 shown that inducing shoot regeneration from hypocotyls, cotyledon and leaf explants using BA has proved more difficult than apex explant. It is very important to choose the right shoots for the study because it will affect the results.

During the culture period, when high level of cytokinins (BA) was used in the media, it will encourage the growth of axillary buds and reduce the apical dominance. From one apex, several unrooted shoots can be produced. The unrooted shoots can be separated and recultured again or it can be rooted by transferring into a different medium (George and Sherrington, 1984). BA also promotes cell division; shoot multiplication and axillary bud formation while inhibiting shoot development (Sutter, 1996).

When concentration of BA are 5.0 μM or higher (10.0, 20.0 and 40.0 μM) there is no rooting can be observed. It is due to the inhibition root formation by high concentration of cytokinin (BA). High concentration of BA will prevent root growth.

At 5.0 and 10.0 μM BA, low number of axillary shoots can be seen due to condition of the explants used. This happen when small shoots were used instead of

bigger and vigorous shoots. Roots were produced when the plants were cultured in the media with hormone concentration of BA lower than 1.0 μM. No roots can be seen when the plants cultured in media with hormone concentration of BA higher than 1.0 μM.

5.2.3 Absence of BA

The growth of shoots and roots of the explants will be affected when cultured on media with increasing concentrations of auxins (NAA). And at higher concentrations of NAA, low numbers of multiple shoots were seen and some produced only one shoot. The shoots were grown slowly and the leaves were curly instead of straight and protruding. A few roots were established when the concentration of NAA was equal or less than 0.5 μM.

According to George and Sherrington, 1984, the synthetic auxins are phytotoxic at high concentration to broad leafed plants and in chronic auxin treatment, root initiated by an auxin (particularly NAA) most possibly failed to grow and also suppressed shoot growth.

5.2.4 BA and NAA in combinations

In media where both BA and NAA were used in combinations, at low concentrations of BA and increasing concentration of NAA, the numbers of shoots produced were decreased accordingly. While at low concentrations of NAA and increasing concentrations of BA, results showed that number of shoots increased but

were stunted in appearance. However, the highest number of shoots produced (5.4±1.95 shoots per explant) was not superior compared to media supplement with 1μM BA alone. There were twelve combinations giving high mean value between 4 to 6 shoots per explant.

At high concentrations of BA and NAA, the morphology of shoots showed abnormality. The plants appeared swollen especially at the petiol area.

From the study, it showed that all of the explants (100%) cultured in the different media produced shoots but differed in the numbers and size of shoots. The study also shows the significance of BA and NAA interactions. It was observed that the concentration of BA used affected the number of shoots differently at each level of NAA and vice versa for NAA at each BA concentrations. Conventionally, high cytokinin to auxin ratio promoted growth of multiple axillaries shoots. However in this study, cytokinin alone (BA) supplemented media produced highest number of shoots.

For micropropagation of *Cryptocoryne lucens*, the optimum PGR combination is 20 μM BA and 0.5 μM NAA and culture on Linsmaier & Skoog media giving 7.7 shoots/explant (Kane *et al*, 1990). This could be due to the influence of endogenous hormones which might have influenced the ratio value and shoot formation.

From the study, explants originating from shoot tips and cultured in MS media with hormone concentration of 1.0 μM BA for forty (40) days cultured appears to be a suitable condition for micropropagation of *Cryptocoryne* x*willisii* giving 6.8±1.8 shoots/explant with some rooting. The results for optimum PGR combination for C. x*willisii* are not accordance to Kane *et al*, 1990 because the media is species dependent.

In this study, all of the explants cultured in the different PGRs concentrations and combinations, produced shoots (100%) but differed in the numbers and sizes of shoots. From the study, tissue culture of *C.* x*willisii* in MS medium with 1.0 μM BA

alone was the optimum formula for producing 6.8 ± 1.75 shoots per explant. Kane *et al* 1990 reported that for micropropagation of *Cryptocoryne lucens*, the optimum media was Linsmaier and Skoog 1965 media (LS) with 20μM BA and 0.5μM NAA producing 7 shoots per explant. It was also observed in the study that all of explants cultured in the different media produced shoots but differed in the number and size of shoots.

From analysis of variance (see Table 3.2), hypothesis null that is no interaction between BA and NAA hormone is rejected. The results for this factorial experiment showed that BA and NAA interact significantly. BA affected the number of shoots differently at each level of NAA and the vice versa for NAA at each BA concentrations.

Conventionally, high cytokinin to auxin ratio promoted growth of multiple axillary shoots. However in this study, cytokinin alone (BA) supplemented media produced highest number of shoots. This is in agreement with worked published by Kane *et al* 1999. This could be due to the influence of endogenous hormones which might have influenced the ratio value and shoot formation. The developmental pathway for *C.* x*willisi* as shown in Plate 3.3.

5.3 Phylogenetic Study of Selected *Cryptocoryne* sp. Of Malaysia and Sri Lanka Using IRAP Analysis

There are more than 60 species of Cryptocoryne in the world and they are widely distributed in Tropical Asia. In Malaysia, there are 19 species of Cryptocoryne (Jacobsen, 1985) and in Sri Lanka there are 8 species of Cryptocoryne reported (Bastmeijer, 1997).

It is very difficult to differentiate the Cryptocoryne by morphology unless by flowering. Unfortunately, most of the Cryptocoryne did not produce flowering and some Cryptocoryne only flowering seasonally. In this study, molecular markers are used to study the phylogenetic relationship between selected Malaysian Cryptocoryne and local cultivated Sri Lanka Cryptocoryne.

IRAP techniques are chosen for the phylogenetic study because 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).

Results form this study showed that the degree of polymorphism of the IRAP products using IRAP primers in Cryptocoryne was high (87.8%, Table 4.3), similar to that reported for many plants such as that observed within sixteen species of Musa (Teo *et. al*, 2005; Kalendar *et. al*, 1999). Table 4.2 shows that from forty-five primer combinations, only seven primer combinations gave successful amplification and high polymorphism.

The results of the phylogenetic grouping using the IRAP data suggested that *Cryptocoryne sp.* could be subdivided into 3 major groups and 5 different subgroups.

Group I consists of *C. auriculata, C. nuri, C. nuri* (Raub) and *C.sp*. (1). All Crypts in this group were Peninsular Malaysian plants. *C. sp.* (1) was also collected from an area in Pahang, but the species is yet to be identified.

Cryptocoryne in Group II were divided into three subgroups, subgroup I including *C. uenoi* and *C. ferruginea* and both were found in Sarawak, subgroup II consisted of *C. xwillisii, C. xwillisii* (tissue culture) and *C. parva,* and subgroup III contained *C. wenthi*, *C. wenthi* Brown and *C. wenthi* Green. Subgroup II and III constituted plants origin from Sri Lanka. The *C. xwillisii* and *C. xwillisii* (tissue

culture) falls under the same group and it showed that the tissue culture plant is the same as the conventional propagation.

The last group consist of two subgroups: subgroup I and subgroup II,. *C. shulzei, C. keei* and *C. affinis. C, striolata, C. purpurea* and *C. pallidinervia* were in subgroup I while Group II contained the *C. cordata* and *C. lingua*. The species in subgroup I did not fit under geographical distribution but for subgroup II, *C. cordata* and *C. lingua* fall under the same geographical distribution because both are found in Sarawak.

Suggestions for future studies are more IRAP markers should be used for analysis to get better results of the phylogenetic and more samples of other Cryptocoryne species to see the relationship between the species and the geographical distribution.

Applications of retrotransposon markers have previously been applied in the study of banana (Teo *et. al*, 2005), cereals (Kalendar *et. al*, 1999; Kalendar *et. al*, 2000) and Poaceae (Baumel *et. al*, 2002) and for the study here are the first application for aquatic plant especially for Cryptocoryne. The result suggests that this approach may be useful for resolving the evolutionary relationships between the Cryptocoryne and may strengthen the taxonomic differentiation and geographical distribution of the different species. It should be noted however that a much larger number of replicates, different samples as well as number of markers used would increase the accuracy of the findings. In their study Teo *et. al*, (2005) used 9 number of markers and 81 combinations of markers to differentiate 3 subgroups of plants which correlated with 9 species of Musa. This data however provides good preliminary evidence for the evolutionary differential of the Cryptocoryne sp.

The results also showed the universality of using retrotransposon-based markers. In this study the primers were develop from retrotransposon markers isolated in banana (Teo *et. al*, 2005).

5.4 Determination of Lethal Dose LD_{50}

Mutants from vegetative propagated plants (asexual plants) are difficult to obtain. Usually explants used are the tuber, cutting plants and shoots.

In the study, all regenerants from shoot explants were showing necrosis when exposed to high dosage of gamma irradiation. Only when the regenerants were subject to lower dose of the gamma ray, the LD_{50} can be achieved. The LD_{50} in the study was 25 Gy and the treatments for mutagenesis are between 0 Gy to 30 Gy. Only low dosages of gamma rays were needed to establish LD_{50} such as on budsticks in lemons, LD_{50} is 50 Gray (Gulsen *et al*, 2007).

After treatment, some of the new shoots of the regenerants showing differences in morphology, some showing necrosis and some did not show any changes. A difference in morphology, which is stably expressed, is called mutant.

These shoot explants consist mostly of meristem cells and it will produce diplon competition. Diplon competition is competition between normal cells and mutant cells at multi cells chimera tissues cause by the mutagen treatment. The damage on mutant cells perhaps causes by abnormal mitosis and retarded the growth. Active normal cells surrounded the mutant cells and finally the phenotype of the mutant cannot be expressed (Chai *et al*, 1990). One way to increase the frequency of induced mutation is through mutagenesis treatment carried out on new shoots from tissue culture explants. These new shoots may derive from either one or a few cells.

5.5 Evaluation Of Gamma Irradiated Plants

In the present study, acute irradiations were given to 2000 explants. Many variants were established after irradiation. Variants produced such as deformation of leaves, light green leaves colour, small and spiral leaves, pink petiole, albino plants, dwarf plants and taller plants. The variants are the M_1 generation. All variants were selected, separated and subcultured individually (M_2). All variants reverted back to normal plants after the second subculture (M_2) except for two types of mutant, D1 (dwarf) and G1 (tall).

The D1 and G1 variants were subcultured until M_4. After the M_4, the two variants are stable and are called mutants. Only 0.1 % irradiated explants turn into mutant plants. The first mutant, D1 is smaller in sizes compared to normal plants (C) including the length and the leaves sizes. The colour of D1 was light green. The second mutant, G1 was bigger in sizes compared to normal plants (C) including the length and the leaves sizes, which were brownish in colour.

5.6 IRAP Analysis For Identification Of Sports (Clonal Mutation)/Mutant

To further characterise morphological differences between the mutant plants, differentiation was also attempted using molecular markers. This approach was carried out to investigate the utility of DNA markers for differentiating between mutant and parental phenotypes. DNA markers have been used in the characterization of variation in many plant species after induced mutation including Hinoki cypress (Ishii *et al*, 2003), soybean plant (Cimen *et al*, 2004) and apple (Kristiina *et al*, 2006) , Among the DNA markers used are RAPD markers (Ishii *et al*, 2003), (Cimen *et al*, 2004) and TRIM retrotransposon (Kristiina *et al*, 2006).

In this study, IRAP markers were used to distinguish the D1 and G1 genomes from normal *C.* x*willisii* genomes. IRAP has the advantages over other markers in that it detect high levels of polymorphism with out DNA digestion, ligations or probe hybridization and it also reliable robust (Kalendar et al., 1999), (Teo *et al*, 2005).

The analysis revealed two unique polymorphic bands (225 bp and 320 bp) using Nikita primer for the D1 mutant and revealed two distinct polymorphic bands (240 bp and 300 bp) using a combination of 3'LTR primer and LTR 6149 primer for the G1 mutant (Figs xxx) that were not amplified in the normal plants. The results suggest that IRAP may be useful for early selection of mutants after treatment and can be further used for identification for fingerprinting of potential new varieties. The latter would be a useful application for commercial use in varietal identification and tracking. It could also be used to track the stability of the mutations in subsequent generations.

In this study although the markers could differentiate between the two mutants, it would be too preliminary to correlate the markers with the observed phenotypic characteristics. Work by Ishii et al., 2003 showed that RAPD analysis was unable to detect any variation in band pattern of the hinoki cypress mutants.

The utility of this method for Cryptocoryne should be explored further in future studies using a larger number of replicates, different varieties of mutants as well as testing several generations of the putative mutants for stability.