Faculty of Resource Science and Technology

IN VITRO PROPAGATION OF *CRYPTOCORYNE FERRUGINEA* ENGLER

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Bachelor of Science with Honours
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IN VITRO PROPAGATION OF *CRYPTOCORYNE FERRUGINEA* ENGLER

CHEN MEI YIN

This project is submitted in partial fulfilment of the requirements for the Degree of Bachelor of Science with Honours

(Plant Resource Science and Management)

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In vitro Propagation of *Cryptocoryne ferruginea* Engler

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ABSTRACT

Cryptocoryne ferruginea Engler (Araceae) is an endemic species of Borneo. Many *Cryptocoryne* species are popular as aquarium plants but there is limited information on the successful commercialization of the species from Malaysia. Surface sterilization using 70 % ethanol for 1 minute, 15 % (v/v) Clorox® for 15 minutes and rinsed thrice with distilled water produced the highest percentage of contamination-free petiole explants at 50 %. For runner explants, 15% (v/v) Clorox® for 12 minutes produced 58% contamination-free runner explants during the first two weeks after culture initiation in Murashige & Skoog (MS) medium. Only 4 explants (4.17 %) runner explants remained uncontaminated after four weeks. Incorporation of Plant Preservative Mixture (PPM™) into MS medium reduced contamination up to 100% in runner explants but only 5 explants (5.21%) were viable for shoot regeneration. The protocol requires further refinement to improve its efficiency. Multiple shoot, leaf and root regeneration used combinations of BAP (0-2.0 mg/L) with or without 0.1 mg/L IBA. Highest mean number of shoots (6.93) and leaves (3.33) per explant was observed in medium supplemented with 1.0mg/L BAP and 0.1mg/L IBA. Addition of 0.1mg/L IBA alone induced highest mean number of roots (2.93) per explant. Different medium (solid or liquid) with or without 0.1 mg/L NAA was used for root induction. Highest mean number of roots (4.25) per explant was induced in liquid medium supplemented with 0.1 mg/L NAA.

Keywords: Araceae, *Cryptocoryne ferruginea*, tissue culture, surface sterilization, shoot proliferation

ABSTRAK

Cryptocoryne ferruginea Engler (Araceae) merupakan spesies endemik daripada Borneo. Cryptocoryne merupakan tumbuhan perhiasan dalam akuarium namun, maklumat penggunaan komersial Cryptocoryne daripada Malaysia amatlah kurang. Pensterilan permukaan paling berkesan untuk batang (50% bersih) pada 2 minggu pertama meliputi pensterilan dalam 70 % ethanol selama 1 minit, 15 % (v/v) Clorox® selama 15 minit dan air steril sebanyak tiga kali. Eksplan runners yang disteril di dalam 15 % (v/v)) Clorox® selama 12 minit menghasilkan 58% eksplan bersih pada dua minggu pertama dikulturkan di dalam media Murashige & Skoog (MS). Hanya 4 eksplan runners (4.17 %)dapat menghasilkan pucuk. Penambahan 'Plant Preservative Mixture' (PPM™) dalam media berjaya mengurangkan kontaminasi di dalam eksplan runners sebanyak 100% tetapi hanya 5 eksplan (5.21 %) menghasilkan pucuk. Protokol ini memerlukan perubahan bagi menghasilkan lebih banyak kultur bersih. Induksi pucuk, daun dan akar menggunakan kombinasi BAP (0-2.0 mg/L) dengan IBA (0, 0.1 mg/L). Min bilangan terbanyak pucuk (6.93) dan daun (3.33) setiap eksplan dicatatkan di dalam media mengandungi 1.0 mg/L BAP dan 0.1 mg/L IBA. Penambahan 0.1 mg/L IBA memberikan min bilangan akar terbanyak (2.93) setiap eksplan. Induksi akar telah mengunakan media MS (cecair atau pepejal) yang ditambahkan dengan NAA (0-0.1 mg/L). Min bilangan akar tertinggi (4.25) setiap eksplan dicatatkan di dalam media cecair ditambah 0.1 mg/L NAA.

Kata kunci: Araceae, Cryptocoryne ferruginea, kultur tisu, pensterilan permukaan, induksi pucuk

TABLE OF CONTENTS

TITLE PAGE		i
APPROVAL SHEET		ii
DECLARATION OF ORIGINAL WORK		iii
ACKNOWLEDGMENTS		iv
ABSTRACT/ABSTRAK		v
TABLE OF CONTENTS		vi
APPENDICES		vii
LIST OF FIGURES		viii
LIST OF TABLES		ix
LIST OF ABBREVIATIONS		x
1.0	**INTRODUCTION**	1
2.0	**LITERATURE REVIEW**	
2.1	Taxonomy	
2.1.1	Botanical description	3
2.1.2	Species identification	6
2.2	Distribution and habitat	7
2.3	Economic importance	9
2.4	*Cryptocoryne* melt	10
2.5	*In vitro* propagation	11
2.5.1	Explants	12
2.5.2	Surface sterilization	12
2.5.3	Contamination problem	14
2.5.4	Medium	15
2.5.5	Plant growth regulators	16

3.0 MATERIALS AND METHODS

3.1 Plant materials 18

3.2 Medium composition

3.2.1 Preparation of MS stock medium 18

3.2.2 Preparation of plant tissue culture medium 19

3.2.3 Preparation of culture media supplemented with Plant Preservative Mixture (PPM™) 19

3.3 Parameters measured 20

3.4 Experimental design and statistical analysis 20

3.5 Establishment of contamination-free cultures

3.5.1 Effects of different concentrations of Clorox® and exposure time 20

3.5.2 Effects of different concentrations of Plant Preservative Mixture (PPM™) 21

3.6 Induction of multiple shoots and roots via direct organogenesis

3.6.1 Effects of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) on shoot elongation and leaf induction 22

3.6.2 Effects of different concentrations of 1-Naphthaleneacetic acid (NAA) and different media on multiple root induction 23

3.7 Induction of callus formation via indirect organogenesis

3.7.1 Effects of 2,4-Dichlorophenoxyaceticacid (2,4-D) on induction of callus formation 23

3.7.2 Effects of 4-amino-3,5,6-trichloropicolinic acid (Picloram) on induction of callus formation 23

4.0 RESULTS AND DISCUSSION

4.1 Establishment of contamination-free cultures

4.1.1 Effects of different concentrations of Clorox® and exposure time 24

4.1.2 Effects of different concentrations of Plant Preservative Mixture (PPM™) 30

4.2 Induction of multiple shoots and roots via direct organogenesis

4.2.1 Effects of 6-benzylaminopurine (BAP) and indole-3-butyric acid (IBA) on shoot elongation and leaf induction 34

4.2.2 Effects of different concentrations of 1-Naphthaleneacetic acid (NAA) and different media on multiple root induction 41

4.3 Induction of callus formation via indirect organogenesis

4.3.1 Effects of dichlorophenoxyacetic acid (2,4-D) and 4-amino-3,5,6 trichloropicolinic acid (Picloram) on induction of callus formation 44

5.0 CONCLUSION AND RECOMMENDATIONS 46

6.0 REFERENCES 48

APPENDICES

Appendix		Page
Appendix A	Murashige & Skoog (MS) medium formulation	52
Appendix B	Two-way ANOVA of between-subjects effects of Clorox® concentration (Bleach) and exposure duration (Time)	53
Appendix C	One-way ANOVA for different PPM™ concentrations on mean number of axenic petiole, runner, and rhizome explants	54
Appendix D	Two-way ANOVA test of between-subjects effects of different BAP concentration and with or without addition of IBA on shoot and leaf induction	55
Appendix E	Tukey's HSD Post-hoc test for effects of BAP on leaf induction	57
Appendix F	Tukey's HSD Post-hoc test for effects of BAP on root induction	58

LIST OF FIGURES

Figure		Page
1(a)	An individual plant of *Cryptocoryne ferruginea*	4
1(b)	The spathe of *C. ferruginea* with a long, twisted tail and opens with a small crack	4
2	Vegetative parts of *Cryptocoryne ferruginea*	
3 (a)	Whitish, halo-like bacterial contamination on a petiole explant cut end	28
(b)	Contamination of petiole explants by fungi with presence of black spores	28
4 (a)	White color of runner explants after 5 days of culture without BAP	35
(b)	Red pigmentation of runner explants after 9 days of culture without BAP	35
(c)	Shoot proliferation and splitting on runner explants in medium with 2.0 mg/L BAP	35
(d)	Red pigmentation on juvenile leaves in medium with 2.0 mg/L BAP	35
(e)	Gradual loss of red pigmentation and further shoot multiplication in medium with 1.0 mg/L BAP in combination with 0.1 mg/L IBA	35
(f)	Root proliferation when cultured in medium with 0.1 mg/L IBA	35
5 (a)	Induction of roots in plantlet cultured in liquid MS medium with 0.1 mg/L NAA	43
(b)	Elongated root in plantlet cultured in solid MS with 0.1 mg/L NAA	43
6 (a)(b)	Yellowish and clear callus formed at the cut edges of petiole explants in full strength MS medium supplemented with 2.0 mg/L 2,4-D at 4 weeks after culture initiation	43

LIST OF TABLES

Table		Page
1	Treatments of surface sterilization for petiole, runner, and rhizome explants	19
2	Shoot multiplication treatments using BAP alone or in combination with IBA	22
3	Mean number of axenic, contaminated, and damaged petiole, runner, and rhizome explants after surface sterilization with different sterilization methods and cultured in ½ MS medium recorded at 2 weeks after culture initiation	26
4	Mean number of axenic, contaminated, and damaged petiole, runner, and rhizome explants after culturing in different concentrations of PPM ™ added into ½ strength MS medium and recorded at 2 weeks after culture initiation	31
5	Comparison of means for number of shoots, leaves and roots produced per replicate after culturing in ½ strength MS medium supplemented with different concentrations of BAP alone or in combination with 0.1 mg/L IBA, recorded at 3 weeks after culture initiation	38
6	Effects of BAP on leaves regenerated from explants based on Tukey's HSD test	40
7	Effects of BAP on roots regenerated from explants based on Tukey's HSD test	40
8	Mean number of roots regenerated per explants after 2 weeks of culture in different media types and with or without addition of NAA	41

LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
2iP	6-γ,γ-dimethylallylaminopurine
ANOVA	Analysis of variance
BAP	N^6-benzylaminopurine
GA3	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kinetin	6-furfurylamino purine
LS	Linsmaier and Skoog medium
MS	Murashige and Skoog medium
NAA	1-Naphtaleneacetic acid
Picloram	4-amino-3,5,6-trichloropicolinic acid
PPM™	Plant Preservative Mixture
TDA	Thidiazuron

1.0 INTRODUCTION

The genus *Cryptocoryne* belongs to the aroid family, Araceae. The common names for these aquatic plants are tropical-bog, kiambang batu (Sarawak Malay), teron anum (Melanau) and kelatai (Iban), keladi laut or bakong (Samarahan Malay) and hati-hati paya (Peninsular Malaysia) (Simon, Ipor, & Tawan, 2008). *Cryptocoryne ferruginea* Engler is an endemic species of Borneo. This plant was initially found in slow running rivers and streams in the inner part of the tidal zone in deep shade (Jacobsen 1985). *C. ferruginea* is abundant in Sarawak but has limited distribution (Ipor, Tawan, Seng, Saupi, & Abai, 2007). Many species of *Cryptocoryne* are popular aquarium plants and both local and international demands for these plants are met through mass collection from natural habitats which often leads to significant decrease of their populations. However, the supply of these plants from exporting countries were also reported to be unreliable and there were considerable losses during transportation because of the leaf decomposition condition called "*Cryptocoryne* melt down" (Bryan, 1990, as cited in Kane, Gilman, Jenks, & Sheehan, 1990).

The identification of *Cryptocoryne* is often confusing because many of its species have seasonal flowering and exhibit high plasticity in their vegetative characters such as leaves and hairs. Populations of *C. ferruginea* have a wide range of habitats with distinct morphological variations between intra-specific populations (Ipor et al., 2007). The growth patterns of most *Cryptocoryne* species in their natural habitats are also varied according to the light intensity they receive and water depth (Ipor, Tawan, & Basrol, 2006). Correct identification and detailed knowledge of cultivated aquarium plants are important and useful during introduction of a new species into a market. The information is also essential to determine the proper care and conditions for optimum growth of the plants (Lehtonen & Falck, 2011).

Propagation of *Cryptocoryne* using rhizome in nurseries was reported as slow with limited scale of production. In addition, most *Cryptocoryne* plants require artificial pollination of flowers for seed production (Mansor & Masnadi, 1994). Ipor et al. (2007) further explained that it was very difficult to maintain a sustainable population of living collections in the greenhouse for many years without proper techniques of cultivation. *In vitro* plant propagation through tissue culture is increasingly used for rapid and mass production of commercial freshwater aquarium plants. This method has decreased problems related to over-collection and loses to water-transmitted diseases.

Availability of numerous *in vitro* propagated *Cryptocoryne* species and cultivars in the market suggest that the genus has adaptability to this mass production tissue culture technology (Kauth & Kane, 2006). *In vitro* propagation of *Cryptocoryne* uses several parts of the plant including the rhizome or runners as starting material because these are produced continuously throughout its life cycle and independent of seasonal restrictions. Successful establishment of *in vitro* cultures was reported for *Cryptocoryne wendtii*, *C. nevilli*, *C. becketii*, *C. bogneri*, *C.* wallisii, C. *willsii*, and *C. thwaitesii* (Staritski, 1977, as cited in Kane et al., 1990; Dissanayake, Hettiarachchi, & Iqbal, 2007; Herath, Krishnarajah, & Wijesundara, 2008). Other species included the *C. lucens* and *C. cordata* from Peninsular Malaysia (Sahidin, Othman, & Khalid, 2007; Amirrudin, Ipor, & Aziz, 2007). At present, there are limited reports on the *in vitro* propagation of *Cryptocoryne* species originated from Sarawak.

This study had two main objectives. The first objective was to develop an efficient surface sterilization protocol for *in vitro* propagation of *C. ferruginea.* The second objective was to examine the responses of the plant to growth regulators for induction of adventitious shoots and roots through direct and indirect organogenesis. The propagation protocol could be further modified to propagate other important, rare or endangered species from the genus for conservation, research, and commercial purposes (Collin & Edwards, 1998).

2.0 LITERATURE REVIEW

2.1 Taxonomy

2.1.1 Botanical description

Cryptocoryne or water trumpets belong to the arum family, Araceae. The name of the genus, *Cryptocoryne* originated from the Greek words *crypto* meaning hidden, and *coryne* meaning club due to its spadix which is completely hidden within the kettle. These monoecious plants are erect herbs with underground rhizomes. There are four habit classifications for aquatic plants which are emergent, floating attached, floating unattached, and submerged. Emergent plants grow above the water meanwhile submerged plants grow below the water surface (Dodds, 2002). Most *Cryptocoryne* are generally submersed during high water levels and half-emergent when water levels drop during dry seasons. *Cryptocoryne* blades vary from oval to ribbon in shape while some have markings or blistered textures called bullates, and the margins are either undulating or smooth (Othman, Jacobsen, & Mansor, 2009).

According to Jacobsen (1985), *C. ferruginea* has chromosome number of 2n = 34, identical to a few other species of Borneo such as *C. pallidinervia, C. bullosa* and *C. auriculata*. The rhizome of *C. ferruginea* was described by Jacobsen (1985) as short and rugged while the submerged ones were often thin with long internodes. The runners were also long and slender. Leaves were reported to be green on the upper surface and regularly had slanting, silvery green features (Figure 2). The lower leaf surfaces were usually a paler shade of green and sometimes, a purplish color could be detected. Minute hairs were observed on the lower surface of the leaves and along the margins. The shape of the blade was narrowly ovate to ovate and about 3-12cm long, 1.5-3cm broad, with a truncate to cordate base. Petioles of *C. ferruginea* were recorded up to 5-15cm long but were longest in length when they were continuously submerged under water (Figure 1 a).

The upper part of the spathe was purplish on the outside while the lower part was more whitish (Figure 1 b). The kettle was large and had a caudate limb with rough surface. The collar of the spathe was a prominent black purple. Fruits were syncarp-like with brownish seeds (Jacobsen, 1985).

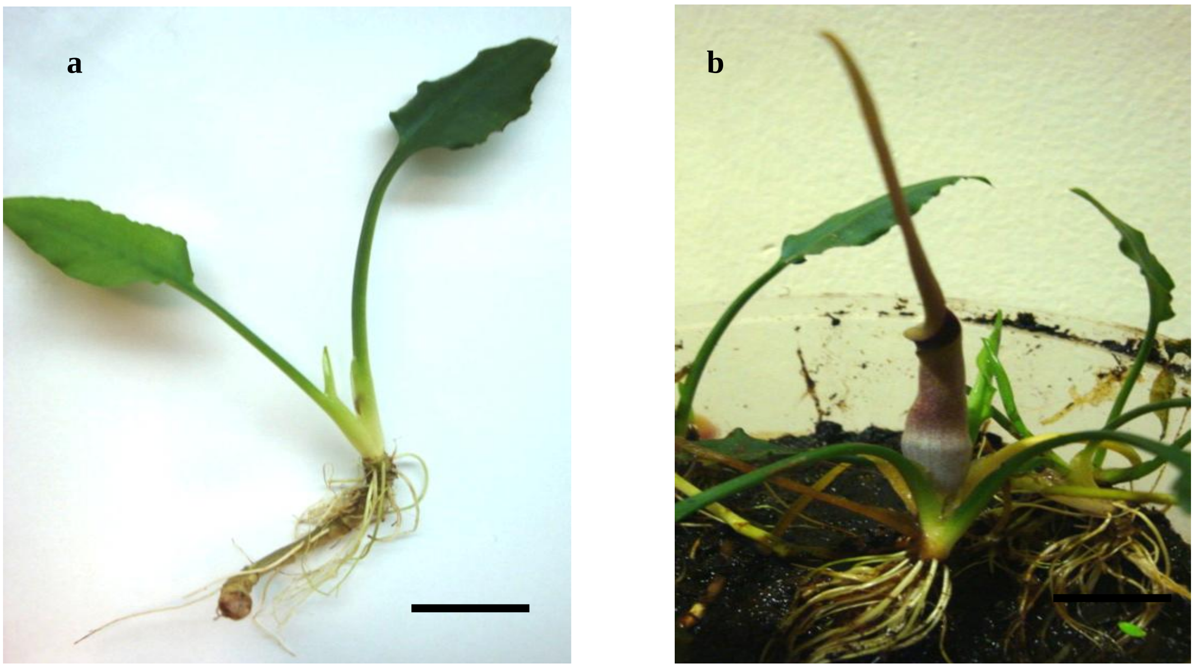

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Figure 1 a) An individual plant of *Cryptocoryne ferruginea*
b) The spathe of *C. ferruginea* with a long, twisted tail and opens with a small crack

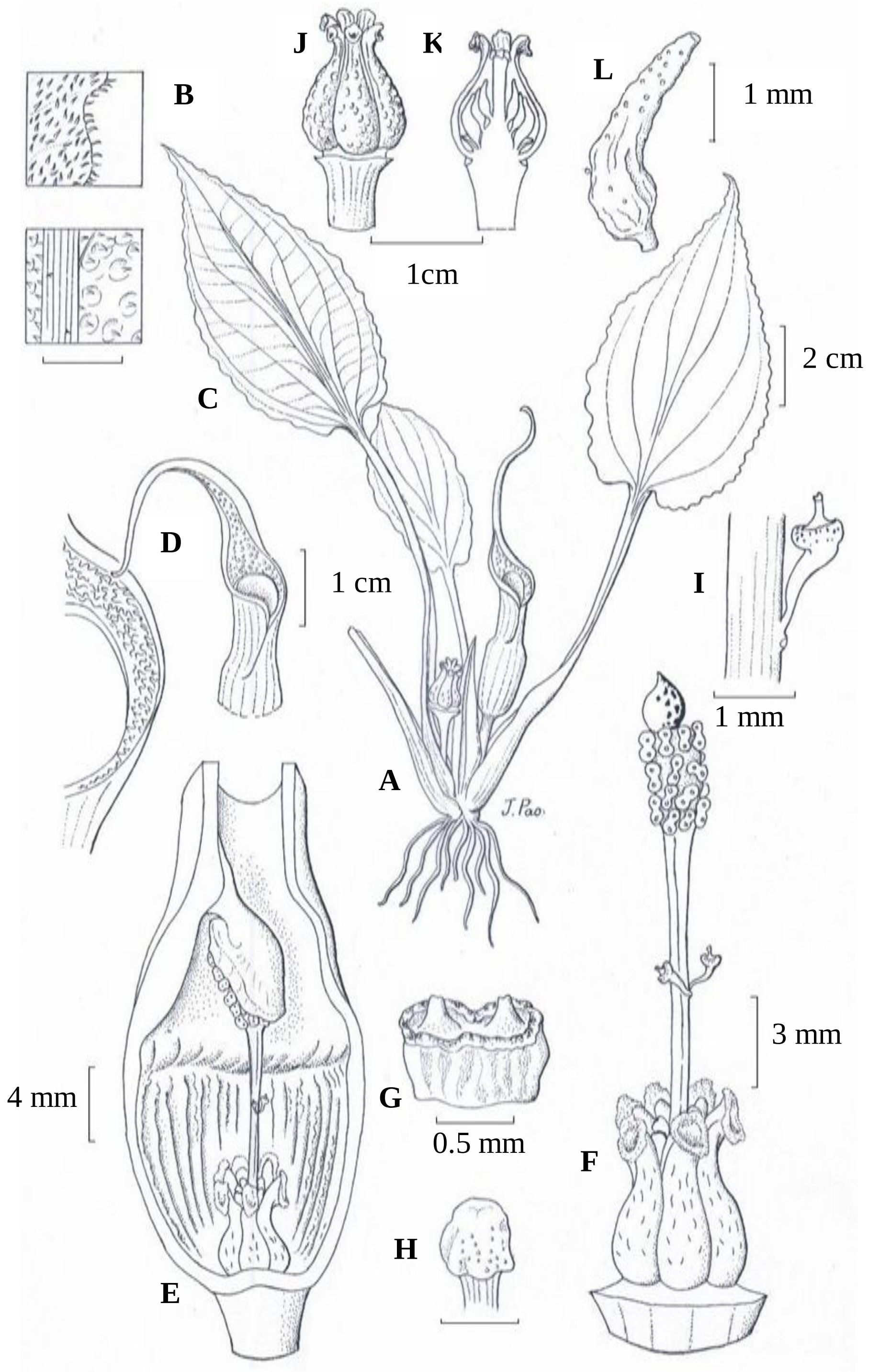

Figure 2: Vegetative parts of *Cryptocoryne ferruginea*. **A**= Whole plant with fruit and inflorescence; **B=** leaf blade, lower surface with hairs; **C**= stomata; **D**= spathe; **E**= kettle; **F**= spadix with female and male flowers; **G**= male flowers with two thecae; **H**= olfactory body; **I**= abnormal structure of male flower with one thecae; **J and K**= syncarpous fruit; **L=** seed (Ipor et al., 2006)

2.1.2 Species identification

The various *Cryptocoryne* species can be differentiated using characteristics of their leaf form, size, and color although the environment is known to exert high variations on these characters. Plants that received more light may produce greener leaves compared to those under overhead shade (Othman et al., 2009). Availability of light is also generally related to chlorophyll amount and photosynthetic rate (Simon et al., 2008). When two or more species of *Cryptocoryne* co-exist within the same river system or water catchment, hybridization may occur to produce hybrids species that could increase problems in identification (Ipor et al., 2007). Jacobsen (1985) reported that the characteristic minute hairs on the lower surface of *C. ferruginea* leaves were sometimes absent. *C. ferruginea* were observed inconsistency in this characteristic which shifted between a complete cover of hairs to complete absence of hairs.

Proper identification of *Cryptocoryne* thus requires detailed examination of their inflorescences. During field visits, it is common to find that the plants were either not in flowering season or were in a vegetative phase so the specific status of the plant may remain unknown unless the site is revisited to check for their inflorescences. Another restraint was that semi-developed flowers have spathe that is often not visible or hidden underwater. Identity of *C. ferruginea* was uncertain for several years because its type specimen had an immature spathe and the species was only recollected in the 1960s (Jacobsen, 1985).

Several *Cryptocoryne* species were treated with gibberellic acid (GA_3) to induce flowering for species identification. The hormone was applied using a foliar spray and the plants successfully flowered after a period of 98-105 days. The treatment was also applied to other ornamental aroids for plant breeding purposes (Kane et al., 1995, as cited in Henny, 1995). This treatment could be applied to induce flowering to confirm the identities of plants collected from the wild before proceeding into selection of suitable cultivation methods.

2.2 Distribution and habitat

Most *Cryptocoryne* species are found throughout Southeast Asia and grow either submersed or emergent (Windelov, 1987). According to Ipor, Tawan, Selamat, and Meekiong (2009), there are at least 18 species in Sarawak which are *C. auriculata* Engler, *C. bullosa* Engler, *C. ciliata* (Roxburgh) Schott, *C. ferruginea* Engler, *C. grabowskii* Engler, *C. keei* Jacobsen, *C. lingua* Engler, *C. longicauda* Engler, *C. pallidinervia* Engler, *C. striolata* Engler, *C. zonata* Engler, *C. uenoi* Y. Sasaki, *C. yujii* Bastmeijer, *C. zaidiana* Ipor & Tawan and *C. fusca* Engler.

According to Jacobsen (1985), *Cryptocoryne* plants have three types of habitats. These are the inner tidal zone containing amphibious life forms, the slow to fast running rivers and streams with mostly aquatic life forms, and the banks of some of the smaller rivers and streams with rheophytic plants. Sexual propagation of *Cryptocoryne* is more frequent in rheophytic habitats and those with amphibious life forms. At lower water levels or when the water current is slow, the plants can successfully flower and undergo pollination to form fruits (Othman et al., 2009).

Populations of *C. ferruginea* have a variety of habitats ranging from slow running rivers and streams in the inner part of the tidal zone, streams above the tidal zone, to a lowland swamp forest. Ipor et al. (2007) reported 4 records of *C. ferruginea* in the Samarahan Division of Sarawak. This species was considered abundant but with restricted distribution. The habitat conditions of these *C. ferruginea* were reported with shallow to moderate water depth and slow to moderate water flow, in alluvial sandy clay soil containing fine gravels (Ipor et al., 2007). The populations at Sabal Kruin were situated at a muddy riverine with frequent flash floods after short tropical rainstorms. In persistent dry seasons, patches of the *Cryptocoryne* in this area sustained in stagnant clear water trapped in ditches (Ipor et al., 2006).

The Balai Ringin populations were described as approximately 300 to 400 meters inward a riverine forest inundated with black peat water. The water there was normally clear with a steady flow. Balai Ringin and Sungai Kerait areas were tested to have acidic water with pH at 5.24 and 5.11 respectively. Meanwhile, water in Sabal Kruin had pH of 4.60. The Sungai Kerait populations occurred along a small stream of Sungai Kerait under moderate deep shading canopy of mature, abandoned rubber plantation but the populations were in small patches because of frequent strong current during heavy rains (Ipor et al., 2006).

Seasonal floods or occasional inundation of *C. ferruginea* habitats were also monitored by Ipor et al. (2006) to benefit the sustainability of the populations. Localities with thick layer of litter were observed to lack *Cryptocoryne.* During inundation of *C. ferruginea* habitats, the layer of litter was swept away when the water was drained which provided a better environment of the population growth. Soil at Sabal Kruin, Sungai Kerait and Balai Ringin were analyzed as poor in nutrient with high percentages of fine particles (69-88%). The source of nutrients for the *C. ferruginea* patches were thus decomposed organic matter from plant biomass debris (Ipor et al., 2006). Ipor et al. (2006) later documented two other localities in Sarawak with *C. ferruginea* which were Sungai Bayor at Sungai Sarawak Kanan and a limestone habitat in Serikin, Bau.

Besides that, the population at Sungai Kerait was reported to be threatened by habitat disturbances due to frequent fishing activities using traditional methods by the local people. According to Simon et al. (2008), human activities are one of the causes of decreasing *Cryptocoryne* populations. Rivers in Malaysia are heavily polluted because of agricultural uses such as paddy fields, fish rearing, drainage canals, and deforestation.

2.3 Economic importance

Several species in *Cryptocoryne* have been commercialized as freshwater aquarium plants. Species in the international market include cultivars of *C. wendtii* such as Tropica, Mi Oya, Green and Brown, *C. becketii*, *C. crispatula* var. balansae, *C. parva* and *C. retrospiralis* from Thailand and Sri Lanka. Foliage colors of *Cryptocoryne* range from red, brown to green with either bullate or smooth surfaces with rare species especially sought as collectibles. Successful growth of inflorescences by growers is rare but often desired. It was reported that most *Cryptocoryne* species sold in the United States were collected from natural populations, imported, subdivided into unbranched plantlets, and maintained in tanks before sale (Kane et al., 1990). Other genus from the same family as *Cryptocoryne* (Araceae) such as *Anubias* and *Aponogeton* are also known to be popular aquarium plants (Pradissan & Pongchawee, 2005; Tanpong, Taychasinpitak, Jompuk, & Jompuk, 2009).

There are currently limited ethnobotanical research on the *Cryptocoryne* plants from Peninsular Malaysia, Sabah, and Sarawak. However, *C. ciliata* is a medicinal plant used in the Bagerhat Sadar and Rampal districts in Bangladesh. *C. ciliata* was used to treat skin diseases such as eczema, abscess, acne, boils, scabies, itch, infection, dermatitis, rash, sores, scar and warts, treatment for snake, insect and animal bites, and also as a stimulant or tonic for debility (Molik et al., 2010). *Cryptocoryne* plants were also used by local physicians in Sri Lanka to treat stomach illness and helminthiasis (de Graaf et al., 1986, as cited in Othman et al., 2009).

The genus *Lagenandra* is closely related to the genus *Cryptocoryne*. Studies of chromosome numbers of the members of the Araceae family suggested that *Cryptocoryne* and *Lagenandra* originated from an ancestral genus with basal chromosome number of x= 9 (Petersen, 1989, as cited in Othman et al., 2009).

A study on the bactericidal activity of *Lagenandra ovata* rhizome oil suggested its potential as an antiseptic agent against susceptible organisms. The greenish-yellow oils of methanol extracts of *L. ovata* rhizome inhibited several Gram positive bacteria including *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Proteus mirabilis* and *P. vulgaris.* Inhibition activity towards *E. coli* was almost equivalent to the standard antibiotic, Chloramphenicol (Selvakumari & De Britto, 2007). *Cryptocoryne* plants may have similar bactericidal effect towards susceptible strains.

Cryptocoryne spp. from Ward's Biological, New York was recognized to support mercury-resistant bacteria when assayed by isolation on plate count agar supplemented with 50µl mercuric chloride. Certain bacteria resist mercury toxicity by using the *mer* operon mechanism to reduce ionic mercury to a less toxic gaseous form. There is currently limited information on the amount of mercury *Cryptocoryne* spp. can tolerate and the specific mechanisms to withstand high mercury exposure but mercury-resistant bacteria such as *Pseudomonas* may have contributed to this trait (Caslake, Harris, Williams, & Waters, 2006).

2.4 *Cryptocoryne* melt

The *Cryptocoryne* melt or rot is a phenomenon in which the leaves are known to suddenly "melt" or dissolve into paste after developing transparent areas at the tips. Usually when one *Cryptocoryne* in an aquarium melts, the rest will be affected in the same way (Anon, 2008). There is limited characterization of this leaf decomposition condition but it had caused significant plant losses during its exporting period into other countries (Bryan, 1990, as cited in Kane et al., 1990). Based on aquarist experiences, this occurrence was caused by combinations of a few hypothetical reasons. Firstly, the water conditions were not maintained in which high nitrate levels caused the melt. Secondly, the water conditions such as temperature, lighting or water parameters were changed drastically.