



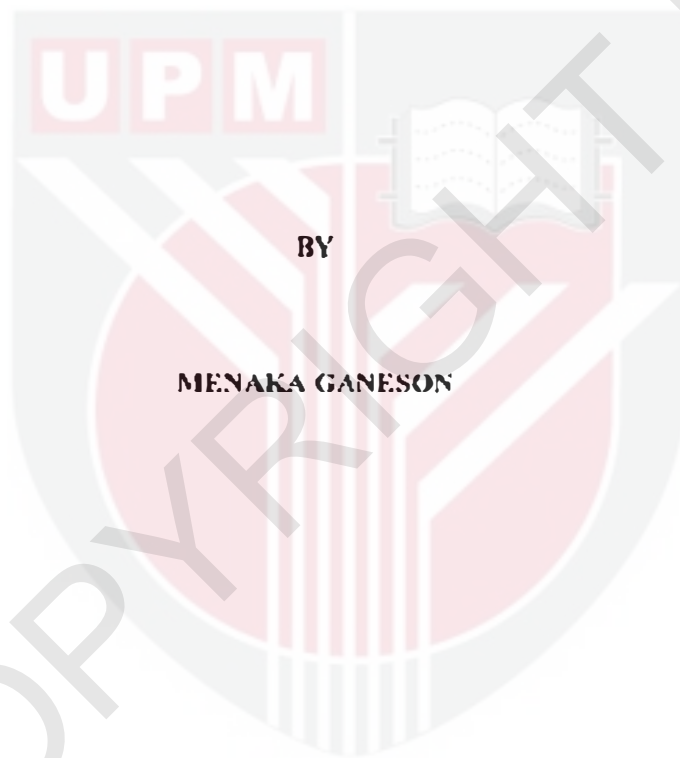
UNIVERSITI PUTRA MALAYSIA

***VEGETATIVE AND REPRODUCTIVE
PROPAGATION OF WATER CHESNUT, *Trapa sp.****

MENAKA GANESON

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FSPM 2008 28**

**VEGETATIVE AND REPRODUCTIVE PROPAGATION OF WATER CHESNUT,
*Trapa sp.***



BY

MENAKA GANESON

**A Project Report Submitted in Partial Fulfillment of the Requirement
for the Degree of Bachelor of Bioindustry Science in the
Faculty of Agriculture and Food Sciences
Universiti Putra Malaysia Bintulu Campus**

2008

DEDICATION

Especially to my father who has been a great source of motivation and inspiration in my life. To my mother and family who have supported me all the way with love and care and
Special thanks to my dear uncles.

ABSTRACT

The water chestnut, *Trapa* sp. is an annual, floating leaved aquatic plant and a valuable food crop. In this study the morphology, seed germination and propagation of *Trapa* sp. were determined for two samples imported from Hong Kong (MDS supermarket) and China (Farley supermarket). Based on morphology the *Trapa* plants from both locations were identified as *Trapa bispinosa* Roxb. The leaves are dimorphic which the floating and submerged leaves. The floating leaves are alternate and in terminal rosette. The floating leaf comprises leaf blade, petiole, and inflated swollen petiole. The length and width of leaf blade, petiole and inflated petiole are distinctly different for both samples. *Trapa* plants from Hong Kong are consistently wider and lengthier than China. The leaf blades are broadly ovate, dentate and rhomboid with prominent veins, serrated at leaf margin, and finely divided into two. The upper leaf blades are glossy bright green and below surface are pale green in colour with leathery. The submerged leaves are opposite, root like and found in 1-2 pairs per node at the stem. The stems are long, submerged, green in colour and 1.81-2.30 mm in diameter. Two types of roots were determined. The anchoring roots are branched and form in 3-12 clusters below hypocotyl. The reddish-brown unbranched roots formed at the upper part of internodes. The flowers are hemapherodite, solitary, hairy peduncle, with 4 white petals, 4 stamens and 1 rounded stigma. The fruits are indehiscent with two horns. Seeds are unequal in size. Seven germination stages were recognized. The first sign of germination stages was emergence of cotyledon at the fruit apex. This followed by emergence of hypocotyls, emergence of plumule, formation of roots on hypocotyl, formation of roots below hypocotyls, development of shoot and young seedling and seed detachment from the seedling. The seeds from both locations took a range of 39-59 days to accomplish the seven germination stages. The life cycle with the eventual death of plants took 3-5 months. The stem cutting propagation only reached matured stage and the life cycle was about 2 months.

ABSTRAK

Trapa sp. (water chestnut) merupakan tumbuhan akuatik setahun bersifat terapung dan mempunyai sumber makanan yang bernilai. Dalam kajian ini morfologi, percambahan benih dan propagasi *Trapa* sp. ditentukan bagi dua sampel yang diimport dari Hong Kong (Pasar raya MDS) dan China (Pasar Raya Farley). Berdasarkan ciri morfologi, tumbuhan *Trapa* dari kedua-dua lokasi dikenalpasti sebagai *Trapa bispinosa* Roxb. Ia mempunyai dua jenis daun iaitu daun terapung dan daun tenggelam. Daun yang terapung tersusun beselang-seli dan membentuk roset. Daun ini terdiri daripada helaian daun, petiol dan petiol yang membengkak. Panjang dan lebar helaian daun, petiol dan petiol yang membengkak mempunyai perbezaan yang ketara bagi kedua-dua sampel. Tumbuhan *Trapa* dari Hong Kong adalah lebih panjang dan lebar berbanding dari China. Helaian daun berbentuk bujur, romboid, hujungnya tajam dengan urat daun yang jelas, bergerigi di tepi daun, dan terbahagi kepada dua. Bahagian atas daun berwarna hijau terang berkilau dan bahagian bawahnya berwarna hijau pudar serta berbulu. Daun yang tenggelam adalah bertentangan, seakan-akan akar dan didapati dalam 1-2 pasangan pada setiap buku di bahagian batang. Batangnya adalah panjang, tenggelam, berwarna hijau dan diameternya adalah 1.81-2.30 mm. Dua jenis akar dikenalpasti. Akar yang bersifat pencengkam bercabang dan terdapat dalam 3-12 kelompok di bawah hipokotil. Akar yang tidak bercabang berwarna coklat kemerahan di bahagian atas batang buku. Bunga adalah hermafrodit, tumbuh secara tunggal, tangkai bunga berbulu, 4 kelopak, 4 stamen dan 1 stigma berbentuk bulat. Buahnya tidak merekah dan mempunyai dwi 'tanduk'. Tujuh peringkat percambahan bijih benih telah dikenalpasti. Peringkat awal percambahan adalah kemunculan kotiledon di bahagian atas buah. Ini diikuti oleh kemunculan hipokotil, plumul, pembentukan akar di atas hipokotil, pembentukan akar di bawah hipokotil, perkembangan pucuk serta tumbuhan muda dan biji gugur. Biji benih bagi kedua-dua lokasi ini mengambil masa 39-59 hari untuk mencapai kesemua peringkat di atas. Peringkat kitar hidup sehingga ke peringkat mati amnya mengambil masa antara 3-5 bulan. Bagi propagasi batang hanya mencapai tahap kematangan dan kitar hidupnya adalah lebih kurang 2 bulan.

APPROVAL SHEET

I certify that this research project report entitled "Vegetative and Reproductive Propagation of Water chestnut, *Trapa* sp." has been examined and approved as a partial fulfillment of the requirement for the degree of Bachelor of Bioindustry Science in the Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Campus.

Dr. Muta Harah Zakaria
Faculty of Agriculture and Food Sciences
Universiti Putra Malaysia Bintulu Campus
(Supervisor)

Assoc. Prof. Dr. Japar Sidik bin Bujang
Faculty of Agriculture and Food Sciences
Universiti Putra Malaysia Bintulu Campus
(Dean)

Date: 24-09-2008

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I would maintain that thanks are the highest form of thought, and that gratitude is happiness doubled by wonder. -G.K. Chesterton

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LIST OF ABBREVIATIONS

°C	Degree celcius
mm	Millimeter
cm	Centimeter
ppt	Part per trillion
s.d	Standard deviation
%	Percentage
NaOH	Sodium hydroxide
DPX	Depex mounting medium
SAS	Statistical Analysis System

CHAPTER I

INTRODUCTION

Water Chestnut, *Trapa* spp. are floating leaved macrophytes found in freshwater water wetlands, ponds, lakes, rivers, and estuaries (Pemberton, 1999; Hummel and Kiviat, 2004; Takanoa and Kadonob, 2005; Hoque *et al.*, 2006a, b). They had been discovered in pre-glacial times and now facing great extinction (Ridley, 1980). They are distributed in various parts of the world (Hoque and Arima, 2004; Arima *et al.*, 2006; Suriyagoda *et al.*, 2006, 2007). The water chestnut contributes to contrast reputation and perception among the authors and countries. They are popular as agricultural product in China, India, and Japan (Hutchinson, 1973; Agarawal and Mohan Ram, 1995; Balgooy, 2001; Hoque and Arima, 2004; Hummel and Kiviat, 2004; Arima *et al.*, 2006; Suriyagoda *et al.*, 2006; 2007). Meanwhile in Europe they are treated as protected species and in United States and other countries they are known as noxious aquatic weed (Oki, 1994; Pemberton, 1999; Hummel and Kiviat, 2004).

Trapa commonly considered as a single genus in the family of Trapaceae (Agarawal and Mohan Ram, 1995; Takanoa and Kadonob, 2005). Some authors previously had classified them in the family of Hydrocaryaceae or Onagraceae (Fassett, 1957; Tanaka, 1976; Ridley, 1980). However Hummel and Kiviat (2004) reported it has been placed under Lythraceae in the order of Myrtales (Purple loosestrife family; Angiosperm Phylogeny Group 1998) due to modern molecular research. The classification of *Trapa* is difficult and often confusing. The species are distinguished according to vegetative characteristic previously but the differences in fruit morphology are the usual used

criteria in classification system (Pemberton, 1999; Takanoa and Kadonob, 2005). Due to variation in fruit shape there is a little agreement and this had designated one, three or up to 30 number of species in *Trapa* (Cook *et al.*, 1974; Pemberton, 1999).

This interesting aquatic plant is heterocotyledon where the seed has two unique and unequal cotyledons: a large cotyledon and the other one is small and scale-like cotyledon (Hutchinson, 1973; Agarawal and Mohan Ram, 1995; Hummel and Kiviat, 2004). The fleshy and starchy large cotyledon is the edible part of water chestnut (Hoque *et al.*, 2006a). Archeological studies had revealed that water chestnut has been used for human consumption since Neolithic ancient times (Nicholson and Evelyn, 1969; Hummel and Kiviat, 2004; Karg, 2006). Tanaka (1976) had described in detail the various preparations of edible seed of *Trapa* species. It is also widely cultivated because it is well known for its nutritious and valuable source of food (Agarawal and Mohan Ram, 1995; Hummel and Kiviat, 2004; Arima *et al.*, 2006; Hoque *et al.*, 2006a, b; Suriyagoda *et al.*, 2006, 2007:). *Trapa* usually introduced as an alternative crop in Japan paddy fields (Suriyagoda *et al.*, 2006; Hoque *et al.*, 2006a, b). The nutrient composition of the fruit is about 80% starch, 5% protein and significant amount of vitamins (Hoque and Arima, 2004; Hoque *et al.*, 2006a, b; Suriyagoda *et al.*, 2006, 2007).

In addition, other essential uses of *Trapa* such as for its medicinal values (Hummel and Kiviat, 2004; Hoque *et al.*, 2006a), religious festival dishes (Agarawal and Mohan Ram, 1995; Ahmad and Singh, 1998; Hummel and Kiviat, 2004; Hoque and Arima, 2004; Suriyagoda *et al* 2007) and environmental benefits (Hummel and Kiviat, 2004) also had been discussed.

Cook (1987) reported in *Trapa* the difficulty in vegetative propagation is due to strong apical dominance. Furthermore no appropriate studies were carried on the seed propagation due to the seeds of *Trapa* which are classified as recalcitrant seeds as they lose viability if the moisture content is reduced (King and Roberts, 1980). Hence biotechnological approach was carried out to determine the protocol for micropropagation of *Trapa* (Agarawal and Mohan Ram, 1995; Hoque and Arima, 2004; Hoque *et al.*, 2006, 2007). Hoque and Arima, (2004) and Hoque *et al.*, (2006) described the purpose of micropropagation using *in vitro* techniques is to increase the multiplication of healthy seedlings and to overcome the effect of seed dormancy.

In addition much work and many aspects of *Trapa* had been studied such as seasonal growth and biomass production (Kunii, 1988), floral biology (Kadano and Schneider), germination ecology (Kurihara and Ikushima, 1991) taxonomy (Takano and Kadonob, 2005; Hoque *et al.*, 2005) and morphological characterization (Suriyagoda *et al* 2006; 2007; Arima *et al.*, 2006). However there has been no comparative study done on vegetative and reproductive propagation of water chestnut. Nevertheless the morphological characteristic and seed germination aspect of *Trapa* have not been recorded in detail.

The aims of present study were: (1) to determine the vegetative and reproductive morphology characteristics of *Trapa* (2) to study the seed germination and (3) to determine the propagation through seed and stem cutting.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy

The genus of *Trapa* were placed under Lythraceae although previously were classified into the family of Hydrocaryaceae, Onagraceae or Trapaceae. Jiarui *et al.*, (2000) reported that *Trapa* is morphologically similar to the Lythraceae family by the ovary's partly inferior position and other structures such as the leaves, floral, fruit and seed.

Takanoa and Kadonob (2005) explained that the classification of *Trapa* is difficult to determine due to the treatment of morphological characters differs among the authors. The characters include the floral, fruit and leaf morphology (Hoque *et al.*, 2005) but most of these vegetative characters are similar and variable in size, so the fruit (nuts) morphology which the size and number of spines are basically used to classified *Trapa* (Takanoa and Kadonob, 2005). However Hoque *et al.*, (2005) and Takanoa and Kadonob (2005) had studied the variation of fruit morphology and applied the molecular techniques which is more reliable and informative to classify *Trapa* spp.

Two commonly cultivated species in Asia are known as *Trapa bicornis* Osbeck and *Trapa bispinosa* Roxburgh which are identified with two horns (Nicholson and Evelyn 1969; Ahmad and Singh, 1998; Quattrochi, 2000; Hummel and Kiviat, 2004) and the four spined nut are the *Trapa natans* usually found in Eurasia, Africa and United States (Hummel and Kiviat, 2004). However most botanists now recognize one species *Trapa natans* comprising two varieties: *T.natans* var *natans* L. and *T. natans* var *bispinosa*

Roxb. (Integration Taxonomy Information System, 2003). In Asia the water chestnut is called by various common names as in India: Singhara nut, China: Ling nut, Chi Shih, Shiu Li, Ling Chio, Lao Ling and the English names varies as Water Caltrop, Horn nut, Bull nut and Jesuit's nut (Tanaka, 1976; Pemberton, 1999; Quattrochi, 2000; Hummel and Kiviat, 2004; Ahmad and Singh, 1998). The common name of water chestnut had lead to confusion between *Trapa* and "Chinese water chestnut", *Eleocharis dulcis* (Ahmad and Singh, 1998).

According to Integration Taxonomy Information System (2003) *Trapa* is classified as below:

KINGDOM: Plantae

PHYLUM : Magnoliophyta

CLASS : Mangnoliopsida

ORDER : Myrtales

FAMILY : Trapaceae

GENUS : *Trapa*

SPECIES : *Trapa* sp.

2.2 General Morphology Characteristic

Water chestnut, *Trapa* is an annual (Cook *et al.*, 1974; Benson, 1979; Pemberton, 1999; Ahmad and Singh, 1998; Hummel and Kiviat, 2004; Takanoa and Kadonob, 2005) or short perennial (Cook *et al.*, 1974) aquatic plant.

2.2.1 Vegetative Morphology Characteristic

Floating Leaves

The floating leaves form a whorled or mosaic like rosette around a central stem on the water surface (Figure 1) (Fassett, 1957; Prescott, 1969; Bursche, 1971; Hutchinson, 1973; Cook *et al.*, 1974; Benson, 1979; Pemberton, 1999; Balgooy, 2001; Charles and O'Neill, 2006). The leaves are rhomboid (Bursche, 1971; Hutchinson, 1973; Cook *et al.*, 1974; Balgooy, 2001; Hummel and Kiviat, 2004), sharply serrated or toothed (Fassett, 1957; Cook *et al.*, 1974; Hummel and Kiviat, 2004) dentate margins (Hutchinson, 1973; Oliver, 1979; Hummel and Kiviat, 2004) and broadly ovate (Hummel and Kiviat, 2004). The merged leaves are bright green above and below are yellowish green with visible veins (Hummel and Kiviat, 2004) or reddish green (Ahmad and Singh, 1998). Each of the floating leaf has an inflated, spongy and swollen petiole with air bladders which enable them to float on the water surface (Figure 1) (Fassett, 1957; Prescott, 1969; Hutchinson, 1973; Cook *et al.*, 1974; Benson, 1979; Balgooy, 2001; Hummel and Kiviat, 2004).

Submerged Leaves

At the internodes of long stem, it bears submerged leaves (Figure 1) which are linear, sessile and caduceous (Nicholson and Evelyn, 1969; Hutchinson, 1973; Cook *et al.*, 1974;

Agarawal and Mohan Ram, 1995; Jiarui *et al.*, 2000; Balgooy, 2001; Hummel and Kiviat, 2004). These leaves will abscise, leaving scars on nodes and replaced by pairs of finely branched, green and pinnate plume like structures (Agarawal and Mohan Ram, 1995; Oliver, 1979; Hummel and Kiviat, 2004). However, according to (Prescott, 1969; Cook *et al.*, 1974; Agarawal and Mohan Ram, 1995; Jiarui *et al.*, 2000) these structures were referred as adventitious roots which are photosynthetic and functions to increase gas absorption.

Stem

The stem of the plant is long and lax (Figure 1) (Prescott, 1969), submerged, internodes elongates and unbranched (Jiarui *et al.*, 2000). Nevertheless the stem was also reported may produce branches and terminates in a rosette (Hummel and Kiviat, 2004).

Roots

At the lower stem also unbranched roots can be found which anchors to the substrate (Agarawal and Mohan Ram, 1995; Ahmad and Singh, 1998).

2.2.2 Reproductive Morphology Characteristic

Flower

The flower of *Trapa* sp. is borne in the axils of the floating leaves (Figure 1) (Pemberton, 1999; Hummel and Kiviat, 2004). It is inconspicuous (Cook *et al.*, 1974; Pemberton, 1999) bisexual (Cook *et al.*, 1974; Jiarui *et al.*, 2000; Balgooy, 2001), solitary peduncle (Hutchinson, 1973; Cook *et al.*, 1974; Ahmad and Singh, 1998; Jiarui *et al.*, 2000), axillary (Hutchinson, 1973; Cook *et al.*, 1974; Ahmad and Singh, 1998)

and short stalked (Hutchinson, 1973; Cook *et al.*, 1974). It also has four petals with white petals, four yellow stamens and two fused ovaries (Nicholson and Evelyn, 1969; Hutchinson, 1973; Cook, 1974; Cook *et al.*, 1974; Benson, 1979; Oliver, 1979; Ahmad and Singh, 1998; Hummel and Kiviat, 2004).

Fruit

Fruits with single seeded develop after pollination (Hummel and Kiviat, 2004). After mature the fruit will detached from the plant and sank to the bottom of the water body due to their heaviness (Kunii, 1988; Pemberton, 1999). The fruit or edible nut resemble bull's head or horns (Ridley, 1980), hard (Ridley, 1980; Hummel and Kiviat, 2004), black (Ridley, 1980; Hummel and Kiviat, 2004), woody (Hummel and Kiviat, 2004) with 2 or 4 horns (Nicholson and Evelyn, 1969; Hutchinson, 1973; Benson, 1979; Hummel and Kiviat, 2004). The fruit is also referred as turbinate drupe (Hutchinson, 1973), fleshy and short-lived (Hummel and Kiviat, 2004). Kurihara and Ikushima (1991) discussed that heavy seeds of aquatic plants such as *Trapa* is unique and requires adaptation to environment where persistence is more important than dispersal.

Seed

Seeds were identified without endosperm and the cotyledons are not the same which can be distinguish by size (Hutchinson, 1973; Cook, 1974; Oliver, 1979; Agarawal and Mohan Ram, 1995).

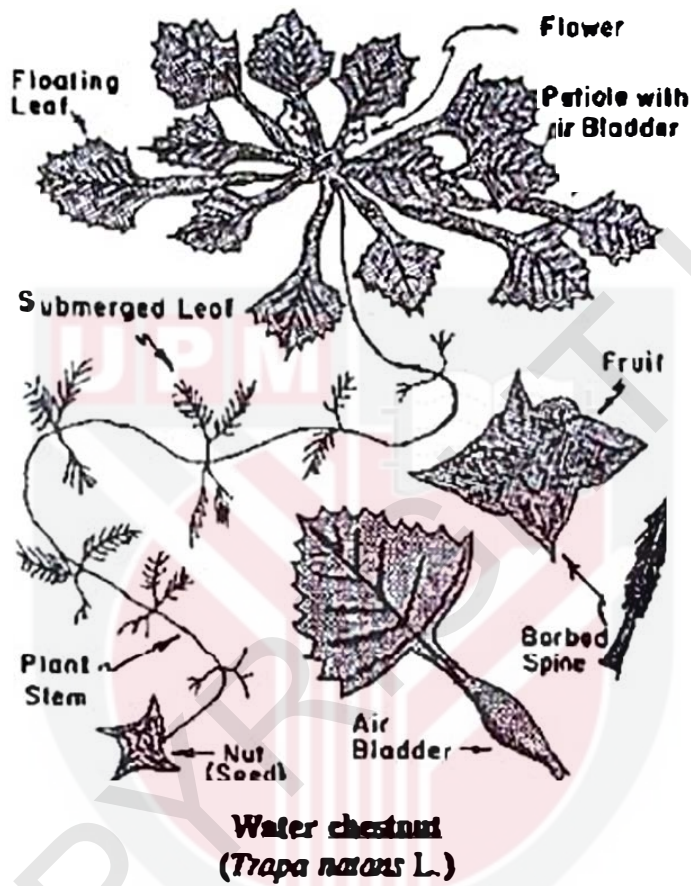


Figure 1: The mature plant of *Trapa* sp. (source from Vermont Water Chestnut Program, 2004)

2.3 Habitat and Ecology of *Trapa* sp.

Water chestnut, *Trapa* is found in the tropical, sub-tropical and temperate zone of the world (Pemberton, 1999; Hummel and Kiviat, 2004; Hoque *et al.*, 2006a; Suriyagoda *et al.*, 2006, 2007). It is native to temperate and tropical Eurasia and Africa (Hummel and Kiviat, 2004). Pemberton (1999) described the native area of *Trapa natans* is from Western Europe and Africa to northeast Asia including Russia, China, and Southeast Asia, through to Indonesia.

According to Takanoa and Kadonob (2005) this aquatic plant is distributed throughout the Old World and naturalized in United States. Water chestnut was also reported once spread naturally in Europe, Asiatic and African areas. In Japan water chestnut is distributed all over the country and usually cultivated in paddy fields (Suriyagoda *et al.*, 2006, 2007). Kurihara and Ikushima (1991) noted this plant in eutrophic lakes, ponds and ditches all over Japan. In India at Kashmir the plant grow in large number (Ahmad and Singh, 1998). Hummel and Kiviat (2004) reported in late 1970s water chestnut was spread in all states of northern India and it grew wild in some regions while others were widely cultivated (Agarawal and Mohan Ram, 1995; Ahmad and Singh, 1998; Hummel and Kiviat, 2004; Suriyagoda *et al.*, 2006; Hoque *et al.*, 2006). The water chestnut was also declining and becoming very rare in certain places especially in Europe (Ridley, 1980; Hummel and Kiviat, 2004).

Water chestnut requires full sunlight (Ahmad and Singh, 1998; Hummel and Kiviat, 2004), nutrient rich with high humus and organic content (Ahmad and Singh, 1998; Hummel and Kiviat, 2004; Karg, 2006), soft or muddy substrate (Pemberton, 1999;

Hummel and Kiviat, 2004; Karg, 2006) and fresh water bodies (Hummel and Kiviat, 2004; Charles and O'Neill, 2006; Iloque *et al.*, 2006a, b). It can grow in water up to 5 m depth but mostly dominates the slow moving and shallow water bodies about 0.3 to 2.0 m depth (Pemberton, 1999; Hummel and Kiviat, 2004; Charles and O'Neill, 2006). Ahmad and Singh (1998) indicated that water chestnut grows best at the depth of 0.5 to 1.0 m and the maximum water depth should not extend more than 1 m.

Trapa favors water with neutral or moderate alkaline pH range of 6.7 to 8.2 (Ahmad and Singh, 1998; Pemberton, 1999; Charles and O'Neill, 2006). pH also affect germination of water chestnut in laboratory (Kurihara and Ikushima, 1991). According to Hummel and Kiviat (2004) earlier the seed germinates faster with a pH of 7.9 to 8.3 followed by pH 4.2 to 5.2 and 5.3 to 7.8. However after two weeks the seedling in acid and alkaline condition died and the moderate acid to neutral substrate form into mature plants (Hummel and Kiviat, 2004).

2.4 Propagation of *Trapa* sp.

Most of the work had been done to develop simple method for the micropropagation of water chestnut. *Trapa* sp. Agarwal and Mohan Ram (1995) had conducted a study in India to develop a simple protocol in micropropagation to overcome the germplasm storage of *Trapa* recalcitrant seeds. In Rajshahi Division of Bangladesh Hoque *et al.*, (2006a) reported the purpose of micropropagation using in vitro techniques is to obtain healthy seedlings and to solve the difficulties on seed dormancy. In addition it would also contribute to large scale production for nursery purpose and manipulate the method for genetic engineering purposes (Hoque *et al.*, 2006a). However during this study callus induction is difficult due to leakage of phenolic substances but the problem was overcome (Hoque *et al.*, 2006a). In vitro shoot production using cotyledonary and nodal explants under various colour illuminations were also studied (Hoque and Arima, 2004). The mixed colour was the best treatment to obtain many shoots proliferation (Hoque and Arima, 2004). Callus induction from water chestnut anther study was done to produce haploid plant for better breeding purposes (Hoque *et al.*, 2006b).

2.5 Importance of *Trapa* sp.

Food

Fossil studies evident had showed that water chestnut's edible seed had been used for food since Neolithic period (Nicholson and Evelyn, 1969; Hummel and Kiviat, 2004; Karg, 2006). The fruit is also treated as valuable agricultural crop in Japan and India (Agarawal and Mohan Ram, 1995; Hummel and Kiviat, 2004; Hoque *et al.*, 2006; Suriyagoda *et al.*, 2006). The white seed is rich in starch, fat and higher in other nutrition values compare to cereals (Karg, 2006) and was widely consumed as staple food in some countries (Cook *et al.*, 1974; Hoque and Arima, 2004; Hoque *et al.*, 2006a, b; Suriyagoda *et al.*, 2006, 2007). Suriyagoda *et al.*, (2007) had stated that water chestnut production would play a small role to overcome the global food problems. It is eaten raw, boiled roasted or preserved in honey or sugar (Nicholson and Evelyn, 1969; Tanaka, 1976).

Beside that the nuts are made into porridge (Nicholson and Evelyn, 1969; Tanaka, 1976). It also used to make coloured powder and dye (Hoque *et al.*, 2006a). Dried water chestnuts were ground to make flour which is subsequently used for making other food stuffs (Nicholson and Evelyn, 1969; Agarawal and Mohan Ram, 1995; Hummel and Kiviat, 2004; Karg, 2006; Hoque *et al.*, 2006a). This nut is always related to Chinese dishes (Nicholson and Evelyn, 1969; Prescott, 1969). In addition the stem and leaves were also cooked and consumed as vegetables (Tanaka, 1976; Suriyagoda *et al.*, 2007).

Religious

Ahmad and Singh (1998) described that water chestnut is considered as 'spiritual horn' and consumed during month of September to celebrate the Festival of the Full Moon. Hence the fruits were sold and are available in markets during this festival time (Hummel and Kiviat, 2004). Beside that, in India, the seeds were used to replace the cereals during religious ceremonies (Agarwal and Mohan Ram, 1995; Hoque and Arima, 2004; Suriyagoda *et al.*, 2007).

Medicine Value

Water chestnut was also appreciated for its medicinal value (Hoque *et al.*, 2006a). The kernels had been used to treat rabies, poisonous animal bite, diarrhea and other diseases (Hummel and Kiviat, 2004).

Ecology Importance

Hummel and Kiviat (2004) had also outlined the importance of *Trapa* in the improvement of water quality as the plant manages to remove nutrients such as nitrogen and reduce eutrophication effect. Suriyagoda *et al.* (2007) reported this plant would solve the global warming to certain level by utilizing the increasing water level. This plant is also able to accumulate on heavy metals which can be used in water purification (Hummel and Kiviat, 2004).

Furthermore, it also provides a large habitat for the invertebrates (David, 2003; Hummel and Kiviat, 2004). David (2003) had reported mass number of macroinvertebrate communities were recorded in *Trapa* beds.

Others Benefits

The water chestnut was also suggested for other beneficial purposes such as for compost, fertilizer, biogas, and paper pulp (Hummel and Kiviat, 2004). Based on the starch richness it was also used as fish feed (Hummel and Kiviat, 2004; Karg, 2006).

Aquatic Weed

Water chestnut, especially the *Trapa natans* was declared as noxious aquatic weed in United States (Pemberton, 1999; Hummel and Kiviat, 2004; Charles and O'Neill, 2006). It had cause problem and was treated as nuisance or pest with increased population (Pemberton, 1999). The high density of floating mats on water bodies limits the sunlight penetration thus causes detrimental effect on aquatic vegetation (Hummel and Kiviat, 2004; Charles and O'Neill, 2006). These causes lost of many animal species and replace the native vegetation (Pemberton, 1999; Hummel and Kiviat, 2004). The effect of water chestnut also associated with reduction in dissolve oxygen (Charles and O'Neill, 2006). The plant is also a main concern because it interrupts human activity for navigation and recreation (Pemberton, 1999; Hummel and Kiviat, 2004). The activities include boating, fishing and swimming (Pemberton, 1999; Hummel and Kiviat, 2004).

Physical Injuries

The sharp and thorny nuts can cause injuries when stepped on (Hummel and Kiviat, 2004; Charles and O'Neill, 2006).

Toxic Substances

Consuming raw water chestnut is common in Asia; however it was reported toxic substances and lead to ingestion problem (Hummel and Kiviat, 2004).

Small population of water chestnut can be easily controlled by hand pulling but the large infestation requires mechanical harvester or aquatic herbicides application (Charles and O'Neill, 2006). Intensive management and control program through mechanical, chemical and biological control were studied to eradicate this plant successfully (Pemberton, 1999; Oki, 1999; Hummel and Kiviat, 2004; Charles and O'Neill, 2006). However the best solution to control still under research and has not been identified yet (Pemberton, 1999; Hummel and Kiviat, 2004).

CHAPTER 3

MATERIALS AND METHODS

The imported seeds from Hong Kong and China were bought from MDS and Farley supermarket respectively during the month of September. The seeds were found abundantly sold at this period of time due to Moon Cake Festival

3.1 Seed Propagation

Thirty eight seeds imported from Hong Kong were cultured on 18th September 2007. Meanwhile the first 58 seeds imported from China were cultured on 14th September 2007 and the other 20 seeds on 12th December 2007. The seeds were cultured by soaked with water in containers and left for 2-3 days at 26°C-28°C under room temperature (Figure 2). The water was changed every 2 days until the hard seed coats were removed from the fruits and the inner coat is can be seen. This curing technique of seed is important to prevent spoilage of the outer shell of the fruits.

After the seeds had germinated, the seeds were separated into new containers or aquariums and the water level were maintained at 15 cm (Figure 3). The germination dates were recorded to calculate the percentage and rate of germination. The germination stages were observed and recorded for both samples until it reaches the seedling stage. The seed detachment periods were also recorded. All the stages were also photographed.



Figure 2: The seeds were soaked in water to allow seed germination.



Figure 3: The germinated seeds were cultured in water in aquarium.

The healthy seedlings were planted in the experimental aquariums with 42 cm length x 26 cm width x 28 cm deep. Before planting that the substrate or soils to be used were tested for the texture and pH of the soil. pH of the water had to be monitored as the plant is sensitive to high acidic and alkaline condition. Two replicates of seeds were planted in each aquarium at the distance of 25 cm of each plant (Figure 4). The water level was maintained throughout the experiment. The aquariums were placed near the window to allow the plant to obtain proper sunlight. The measurement on matured floating leave such as petiole length, petiole width, length and diameter of inflated petiole, blade length and width were measure using Mitotoyo vainer caliper.

Some of the seedlings were transplanted in the pond provided (Figure 5). The pond is located at Latitude 3° 21' 09.5" N, Longitude 13° 09' 19.6" E. Prior to planting weeding was done in the area by hand pulling. Parameters such as the water pH, texture of the soil, temperature (°C), salinity (ppt) and conductivity were obtained. The plants were planted in two rows 30 cm apart, at the distance of 30 cm of each plant and at 0.3 m of depth.

3.2 Stem Cutting

The same procedures were applied for vegetative propagation by using stem cutting in the laboratory. Stem or nodal cutting about 20-30 cm were done and transplanted in the aquarium and containers. The same soil substrate was used. The aquarium and containers were placed near the window to obtain sunlight.



Figure 4: The transplanted seedling in soil substrate at the distance of 25 cm from each plant.



Figure 5: The pond which was used to transplant the seedlings.

3.3 Morphological observation

The vegetative morphology (floating leaves, submerged leaves, roots, and stem) and reproductive structures (flower, fruits and seed) were observed. The length and width of leaves, petiole, inflated petiole, peduncle, fruit and also number of sepals and petals on flower were observed and measured. All measurements were recorded using a ruler and Mitutoyo vainer caliper (accurately measured to two decimals). The morphological features of specimens were observed under the dissecting microscope, noted and photographed.

3.4 Clearing Techniques

Clearing techniques were done to examine internal structure of leaf under microscope. The leaves were cut into 1 cm². Then the leaves were heated about 16° C with addition of 15% of NaOH. The leaves were washed with distilled water and soaked in 5% sodium hypochlorite. After that followed by washing the leaves with distilled water again and dehydrated with alcohol at the different concentration of 50%, 70%, 95% and 100%. The leaves were stained with 1% safranin and placed in clove oil. They were mounted in DPX mountant. Finally the stained leaves can be observed under compound microscope for further identification of leaf structure.

3.5 Herbarium Techniques

The plants were preserved and stored as herbarium samples. The preparations of the herbarium were followed as the steps below:

1. The samples were washed with tap water and arranged in a sheet of paper.
2. The samples were placed between two sheets of papers.
3. Then the samples were put between two sheets of cupboard and two sheets of plywood.
4. A pair of ropes was used to stack the sample.
5. Then the tied samples were placed in air circulating oven at about 40° C for one week.
6. Finally the samples were mounted on herbarium sheet and with a label on the lower right corner of the card.

3.6 Data Analysis

The collected data from both samples were analyzed using SAS 9.1 package and t-test was done to compare the morphological dimension difference between both samples.

CHAPTER 4

RESULTS

4.1 General vegetative and reproductive morphology

Trapa sp. is dicotyledon plants refer to free floating aquatic macrophytes which can be differentiated into vegetative and reproductive components. The vegetative components include the floating leaves, submerged leaves, petioles, stem and roots (Plate 1). The reproductive component comprises of flower, fruit and seed (Plate 2).

4.1.1 Vegetative morphology

Floating Leaves

The floating leaf comprises of blade, petiole and inflated petiole (Plate 3). The measurement for blade length, blade width, petiole length, petiole width, inflated petiole length and inflated petiole width showed significantly different for both locations, Hong Kong (MDS supermarket) and China (Farley supermarket) (Table 1).

Plants grown from Hong Kong imported seeds consistently showed larger and bigger vegetative parameter compare to plants grown from China imported seeds (Table 1).



Plate 1: The general vegetative morphology of *Trapa* sp. n-floating leaves, b- leaf blade, ip-inflated petiole, p-petiole, sb-submerged leaves s-stem and r-roots.



Plate 2: The general reproductive morphology of *Trapa* sp. a-flower, b-fruit and c-seed.

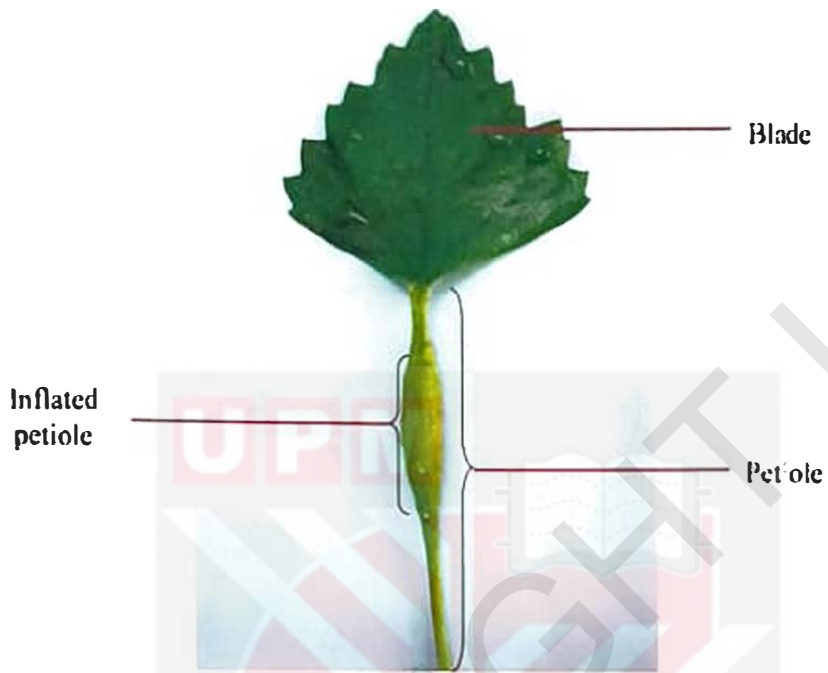


Plate 3: The floating leaf showing leaf blade, petiole and inflated petiole

Table 1: Morphological measurement of floating leaf, *Trapa* sp.

Seed sample	Hong Kong	China
	(MDS supermarket)	(Farley supermarket)
Parameter (mm)	Mean \pm s.d	Mean \pm s.d
	Min-Max	Min-Max
	20 samples	20 samples
Blade Length	28.38 \pm 2.04 ^a (24.36-32.39)	18.47 \pm 2.78 ^b (11.36-20.14)
Blade width	31.30 \pm 2.04 ^a (27.87-38.42)	20.45 \pm 3.18 ^b (12.28-24.50)
Petiole length	50.27 \pm 7.33 ^a (31.01 - 62.25)	42.99 \pm 5.46 ^b (31.09-50.17)
Petiole width	2.11 \pm 0.24 ^a (1.54-2.37)	1.94 \pm 0.38 ^b (1.07-2.33)
Inflated petiole length	14.13 \pm 2.20 ^a (9.73-18.32)	12.18 \pm 1.38 ^b (9.97-14.33)
Inflated petiole width	6.10 \pm 1.37 ^a (3.60-9.08)	3.94 \pm 0.72 ^b (2.87-5.01)

Means with same alphabet are not significantly different (t test, $p \leq 0.05$).

The leaf blade is simple, green in colour with glossy upper surface (Plate 4a). Young leaf blade is reddish-green in colour (Plate 4b). The lower surface of leaf blade is pale green in colour (Plate 4c). The floating leaves are in terminal rosette, whorled structure with alternate arrangement (Plate 4c). The prominent and clear veins below the leaves are visible and very clear.

The blade of floating leaves is broadly ovate in outline, rhomboid and dentate (Plate 5a). At the edges of the leaf margin, it is sharply serrated (Plate 5b) and finely divided into two (Plate 5c). The leaf tip is acute. At the leaf tip the leaves are not divided nor serrated (Plate 5d). The leaves are also leathery at the below surface (Plate 5d).

Each of the floating leaf consists of petiole and an inflated petiole at above the middle of petiole (Plate 6a). The inflated swollen petiole has air bladders which enable them to float on the water surface and allows the foliage to form a typical rosette (Plate 6b). This petiole is hairy which clearly visible (Plate 6c).

Submerged Leaves

The submerged leaves are green and opposite at internodes (Plate 7a). These leaves were in 1-2 pairs (Plate 7b). The submerged leaves were formed at the scars of internodes at the stem after the juvenile leaves abscised (Plate 7c). These leaves are branched and resemble-like roots (Plate 7d).

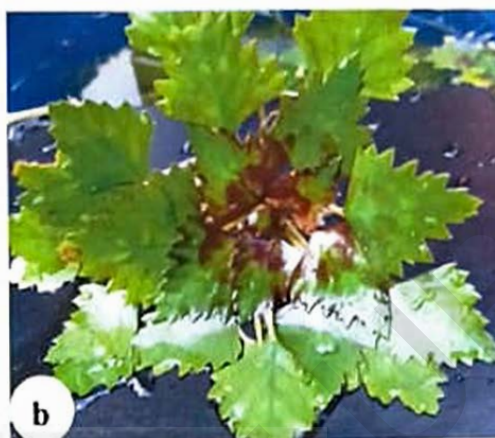


Plate 4: The matured and young floating leaves of *Trapa* sp. a-Adult phase of floating leaves bright green in colour, b-The young phase of floating leaves reddish green in colour and c-The lower surface of floating leaves with alternate arrangement, pale green in colour and prominent veins, Scale bar 2 cm.

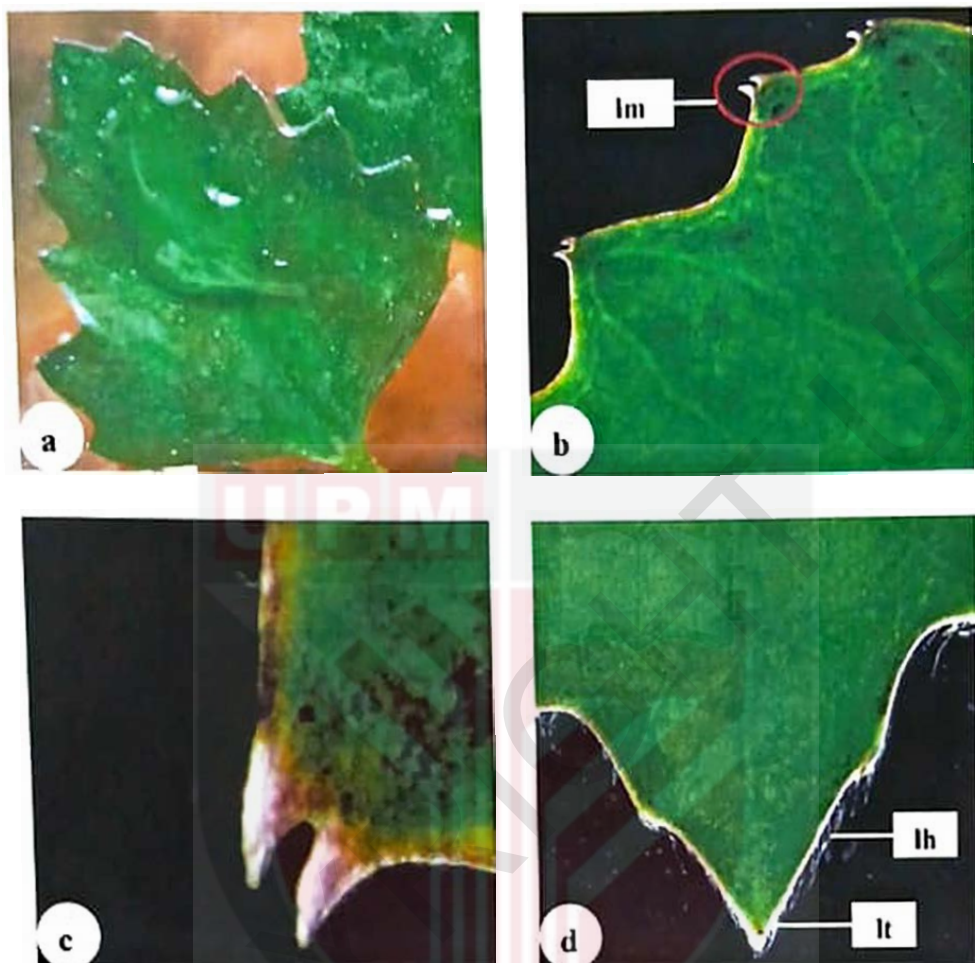


Plate 5: The blade of *Trapa* sp. a-The broadly ovate blade, b-The serrated view at the edges of floating leaf margin (magnification 1.6X), lm-serrated leaf margin, c-The floating leaf is divided into two (magnification 3.2X) and d-the leathery part below the surface of floating leaf magnification 1.6X), lt-leaf tip and lh-leathery.

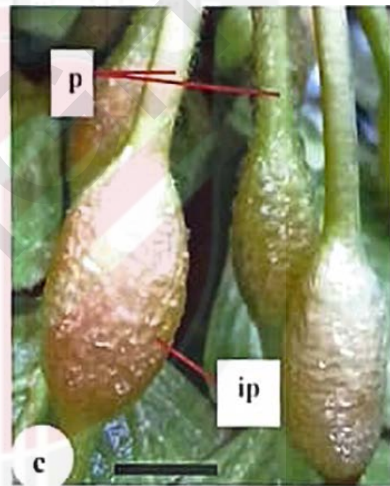
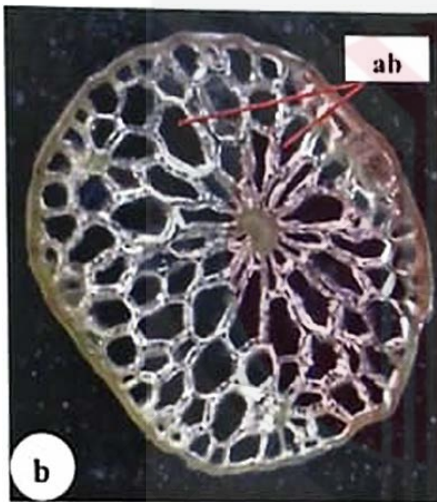
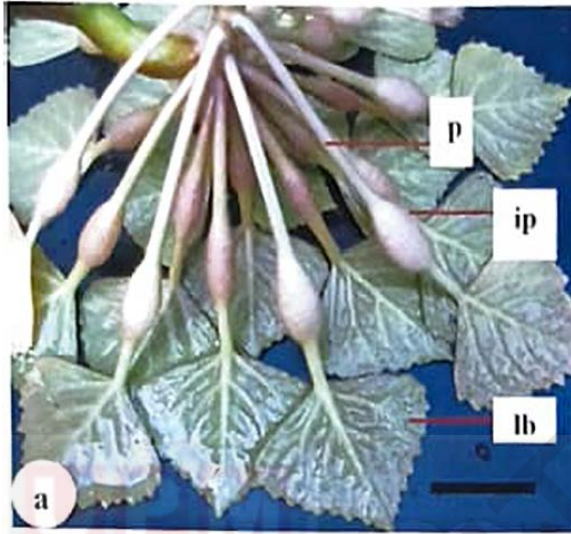


Plate 6: The floating leave of *Trapa* sp. a-The petiole and inflated petiole, b-Cross section of inflated petiole with air bladders (magnification 1.6X) and c-The hairy petiole. Scale bar 1 cm. p-petiole, ip-inflated petiole lb-leaf blade, ab-air bladders. Scale bar 2 cm.

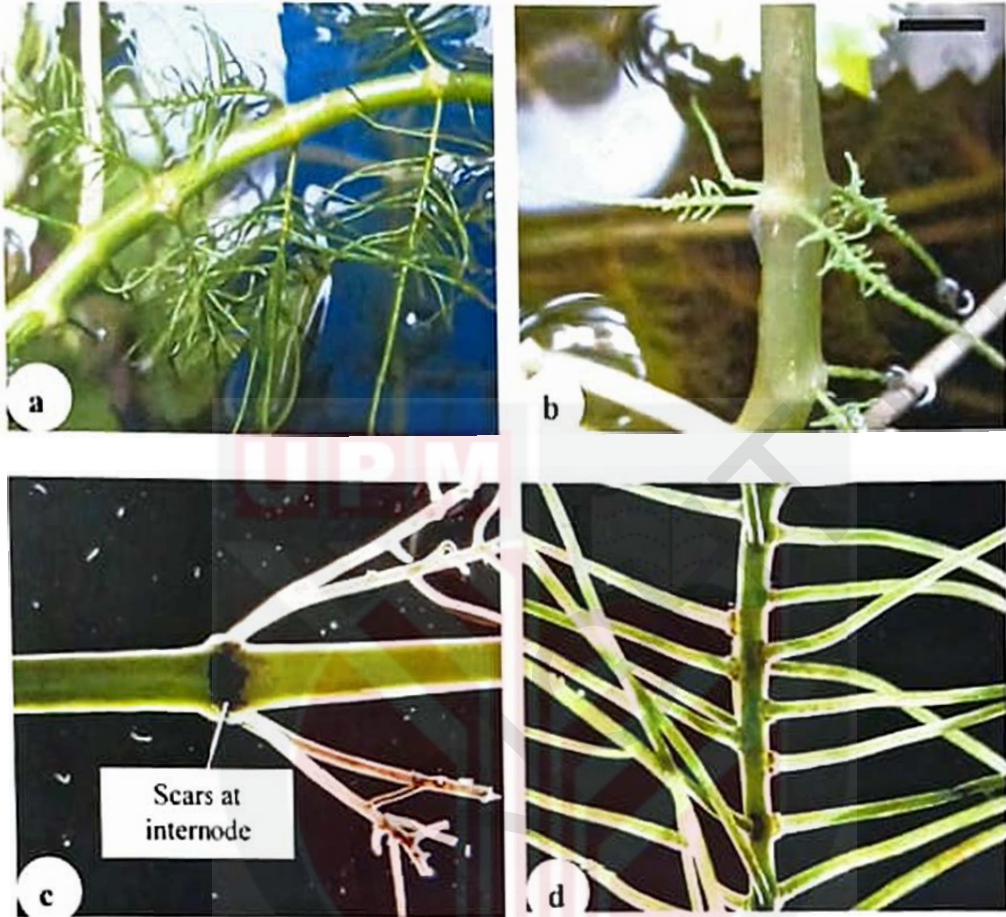


Plate 7: The submerged leaves of *Trapa* sp. a-The green submerged leaves arranged in opposite at the internodes b-The leaves formed in pairs. Scale bar 1 cm. c- Formation of leaves at the scars of internodes (magnification 2.0X) d-The branched and root like submerged leaves (magnification 1.6X).

Stem

The stems are long, slender and submerged with green in colour (Plate 8a). The range of stem width for Hong Kong (MDS supermarket) and China (Farley supermarket) sample was 1.81-2.30 mm and 1.94-2.15 mm respectively. The stem consists of internodes (Plate 8b). The internodes reduced as it reaches the shoot tip where it bears rosette structure of floating leaves (Plate 8c).

Roots

The *Trapa* sp. produces two types of roots. The first roots appear during seedling stage. These roots were green in colour, long, unbranched and formed in clusters of 3-12 at the basal of hypocotyl (Plate 9a). Later it began to branch and form fibrous roots (Plate 9b). The second types of root appear at the upper part of internodes and were unbranched. These long roots were white in colour earlier (Plate 9c) and changed into reddish- brown when the plant matured (Plate 9d).

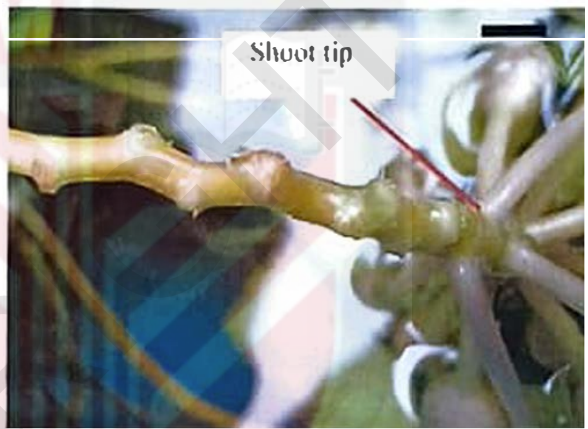


Plate 8: The stem of *Trapa* sp. a-The matured and long, st-green stem, b-Internodes at the stem, in-internode c-Length of internodes reduced as it reached the shoot tip, Scale bar 1 cm.

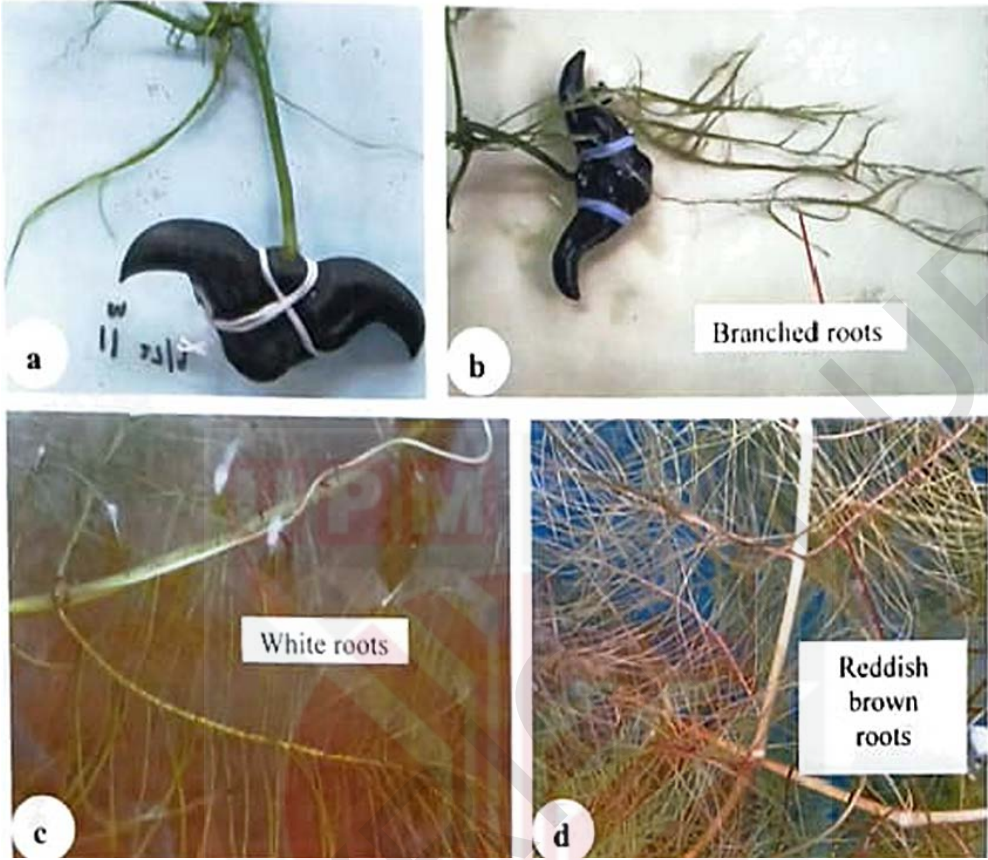


Plate 9: Roots of *Trapa* sp. a-The long, green, unbranched roots, b-Branched fibrous roots, c-White roots at upper internodes and d-The reddish brown roots when matured.

4.1.2 Reproductive Morphology

Flower

The flower is hermaphrodite and solitary in the leaf axils (Plate 10a). The flower emerged from the axils of floating leaves. The peduncle of flower is hairy (Plate 10b). The flower comprises of four sepals, four white petals, four yellow stamens and one stigma (Plate 10c).

Fruit

The fruit is indehiscient (nut), triangular and large with two horns (Plate 11). The fruit is black in colour and hard. The fruits have sharp (Plate 11a) and blunt spine (Plate 11b). At the fruit apex, the barballate structure was observed (Plate 11c). The measurement of fruit height, fruit width and spine length was obtained from the sample imported from Hong Kong (MDS supermarket). The mean of fruit height, fruit width and spine length were 14.13 ± 6.05 mm, 12.20 ± 6.50 mm and 34.81 ± 16.03 mm, respectively (Table 2). The peduncle length mean for mature fruits is 24.77 ± 2.67 mm (Table 2).

Seed

The seed had two unequal size of white cotyledon. The large cotyledon comprised the bulk of the seed while the small scale like cotyledon within embryonal axis emerges out through the terminal of the fruit during germination (Plate 12a). After dissected the internal structures such as small cotyledon within the embryonal axis (Plate 12b), hypocotyl, pericarp and seed coat can be observed (Plate 12c). The seed coat was hard and the pericarp was softer.

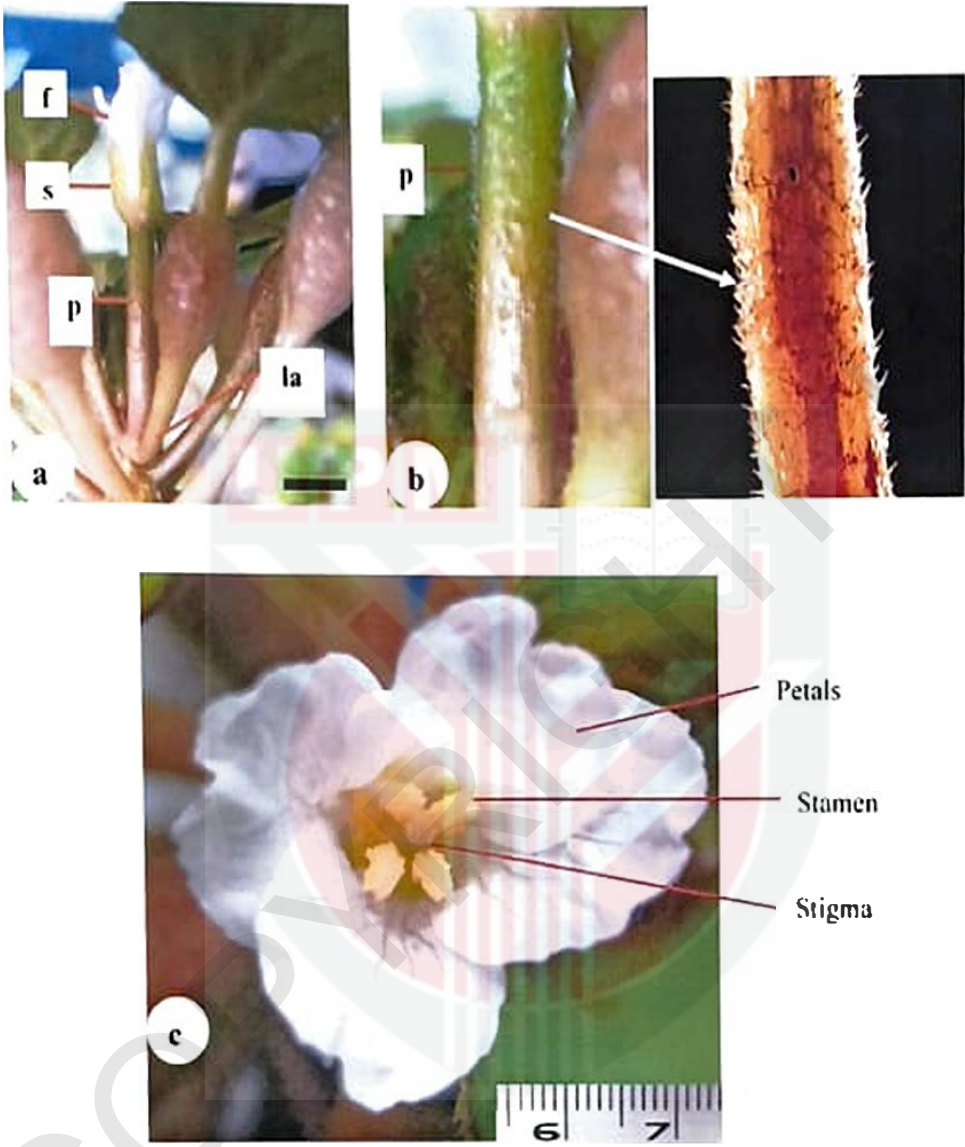


Plate 10: The flower of *Trapa* sp. a-The emergence of flower, Scale bar 1 cm. b-The hairy peduncle (magnification 1.2X) and c-The flower with petals, stamen, stigma. f-flower, s-sepal, p-peduncle and la-leaf axils of floating leaves.



Plate 11: The fruit of *Trapa* sp. a- The fruit with sharp spine, b-The fruits with blunt spine and c-The barbellate structure at fruit apex.

Table 2: Morphological measurement of fruit, *Trapa* sp.

Parameter (mm)	Mean \pm s.d	Min - Max
Fruit spine length	34.81 \pm 16.03	16.19 – 66.43
Fruit width	12.20 \pm 6.50	3.40 – 23.90
Fruit height	14.13 \pm 6.05	5.12 – 25.60
Peduncle length	24.77 \pm 2.67	19.17- 28.46

Data were collected from 16 samples when the fruits are matured. Values denote Mean \pm standard deviation.



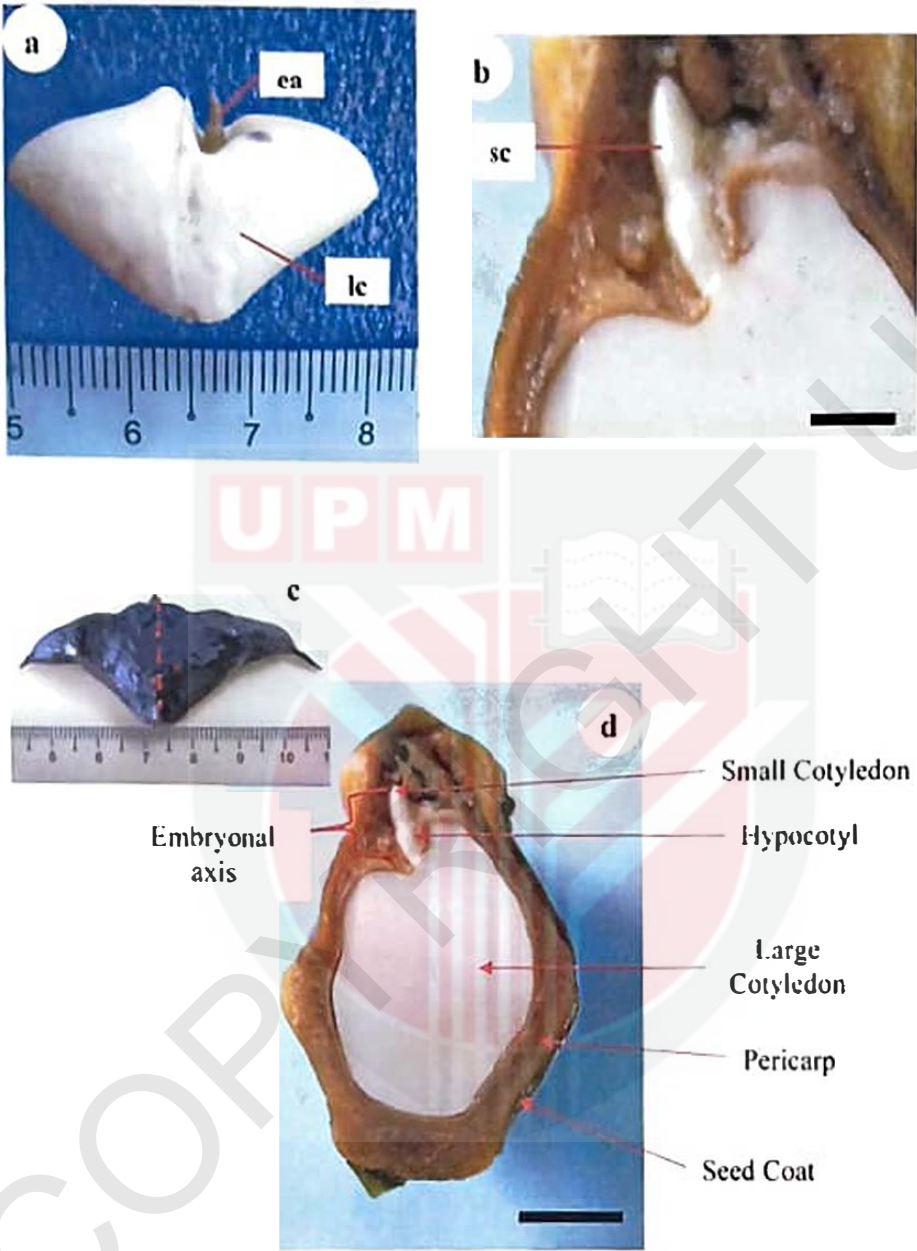


Plate 12: Seed of *Trapa* sp. a-The large and small cotyledon within the embryonal axis, b-The small cotyledon. Scale bar 1 cm, c-The section where fruit was dissected and d-The internal structure of seed after dissected. ea-embryonal axis. lc-large cotyledon and sc-small cotyledon. Scale bar 1 cm.

4.2 Seed Propagation

4.2.1 Seed germination

The percentage germination of seeds in soaked condition under room temperature (26°C-28°C) imported from Hong Kong (MDS supermarket) and China (Farley supermarket) showed 81.25% and 33.33% respectively (Table 3). The germination period for seed imported from Hong Kong (MDS supermarket) ranged from 3-12 days, while seed from China (Farley supermarket) ranged from 2-16 days (Table 3). The germination period (days) and rate of germination (%) for both seed samples were shown in Figure 1. The maximum rate of germination showed more seeds germinated synchronously at that period.

Germination stages

The germination stages of *Trapa* seeds were recognized as (1) emergence of cotyledon, (2) emergence of hypocotyl, (3) emergence of plumule, (4) formation of roots on hypocotyl, (5) formation of roots below hypocotyl (6) developing shoots and young seedling and (7) seed detachment (Plate 13). The range of days taken to achieve the seven sequences of germination stages for both seed sample location from the germination period was shown in Table 4.

Table 3: Seed germination of *Trapa* sp.

Seed Germination	Imported seed from Hong Kong	Imported seed from China
Total seeds cultured	32	78
Germinated seeds	26	26
Percentage of germination (%)	81.25	33.33
Germination period (days)	3-12	2-16

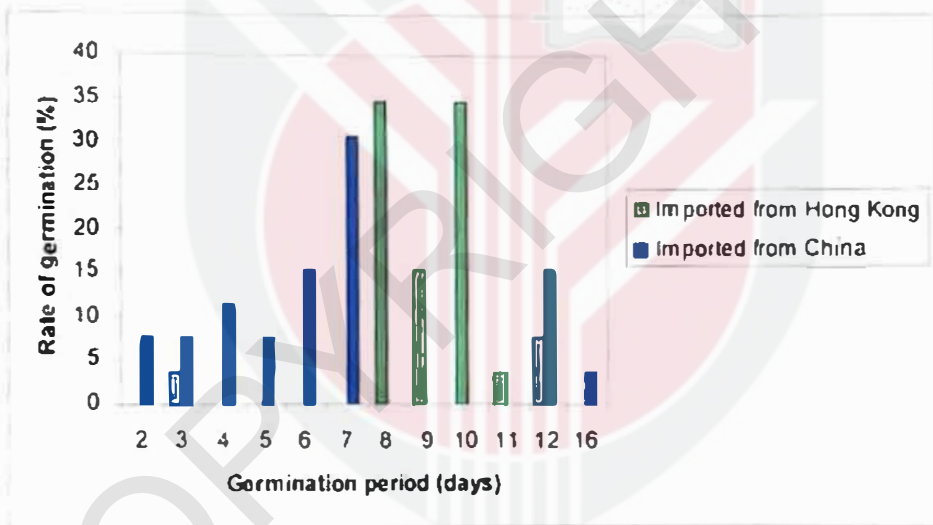


Figure 6: The germination period and rate of germination (%) for both seed sample imported from Hong Kong and China.



Plate 13: The germination stages of *Trapa* sp.; a-Seed of *Trapa* sp. b-Emerge of cotyledon c-Emerge of hypocotyl d-Emerge of plumule e-Formation of roots f-Plumule formation g-Young seedling. s-seed, c-cotyledon, h- hypocotyl, p-plumule, ro-roots on hypocotyls, rb-roots below cotyledon, ps-pioneer shoot, fl-floating leaves, sb-submerged leaves, st-stem.

Table 4: Germination period and germination stages of *Trapa* sp.

Seed sample	Germination stages (days from the germination period)									
	Germination period/ Emergence of cotyledon (days)	Rate of germination (%)	Emergence of hypocotyl	Emergence of plumule	Formation of roots on hypocotyl	Formation of roots below hypocotyl	Developing shoots to young seedling	Seed detachment		
Imported from Hong Kong (MDS supermarket)	3	3.85	3	5	10	8	11-34	51		
	8	34.62	3-4	5-7	6-11	8-14	15-35	53-56		
	9	15.39	3-4	6-7	5-11	9-12	11-36	53-56		
	10	34.62	3-4	5-7	5-12	8-12	12-38	54-56		
	11	3.85	3	6	6	9	11-32	55		
	12	7.69	3	6	6-7	9	15-38	54-56		
Range	3-12	100%	3-4	5-7	6-12	8-14	11-38	51-56		
Imported from China (Farley supermarket)	2	7.69	2	4	5-8	9	11-28	41		
	3	7.69	2	4	8	8-13	13-32	41		
	4	11.54	3	4-7	7-11	9-14	15-32	39-41		
	5	7.69	3	5	8-11	9-10	11-29	39-40		
	6	15.39	2-3	4-7	7-12	9-13	13-29	39-41		
	7	30.77	2-3	4-8	6-12	8-14	13-40	51-59		
Range	12	15.39	2-3	5-8	6-11	7-14	13-40	52-59		
	16	3.85	2	5	8	14	11-36	52		
Range	2-16	100%	2-3	4-8	5-12	7-14	11-40	39-59		
Range from both samples	2-16	-	2-4	4-8	5-12	7-14	11-40	39-59		

The white embryonal axis emerged from germination pit on top of fruit and remained quiescent for 2-4 days (Plate 14a). The small and scale line embryo was indicated as cotyledon which it turned green later (Plate 14b). The green cotyledon elongated and attained a maximum length mean of 24.77 ± 3.71 mm for seeds imported from Hong Kong (MDS supermarket) while the seeds imported from China (Farley supermarket) was 24.99 ± 8.00 mm. The hypocotyl portion emerged (Plate 14c) and elongated simultaneously with the cotyledon within 2-4 days. The hypocotyl attained a maximum mean length of 45.71 ± 11.13 mm and 47.71 ± 11.13 mm for imported seeds from Hong Kong (MDS supermarket) and China (Farley supermarket) respectively.

After growth of hypocotyl, a visible bulge develops below the small cotyledon which indicates the growth of plumule (Plate 14d) within 4-8 days. Later the lip-like cotyledon curved upwards and allows the emergence of plumule (Plate 14e). Then the plumule bore one or two pairs of opposite and decussate leave (Plate 14f).

A few small green roots on the hypocotyl axis developed within 5-12 days after plumule developed (Plate 15a) and increased in length. However in some seeds the roots along hypocotyl developed first before the plumule emerge (Plate 15b). A few roots were also developed below the hypocotyl in range of 7-14 days (Plate 15c). The green roots below hypocotyl elongated rapidly and these roots were also found at the site of plumular (Plate 15d).

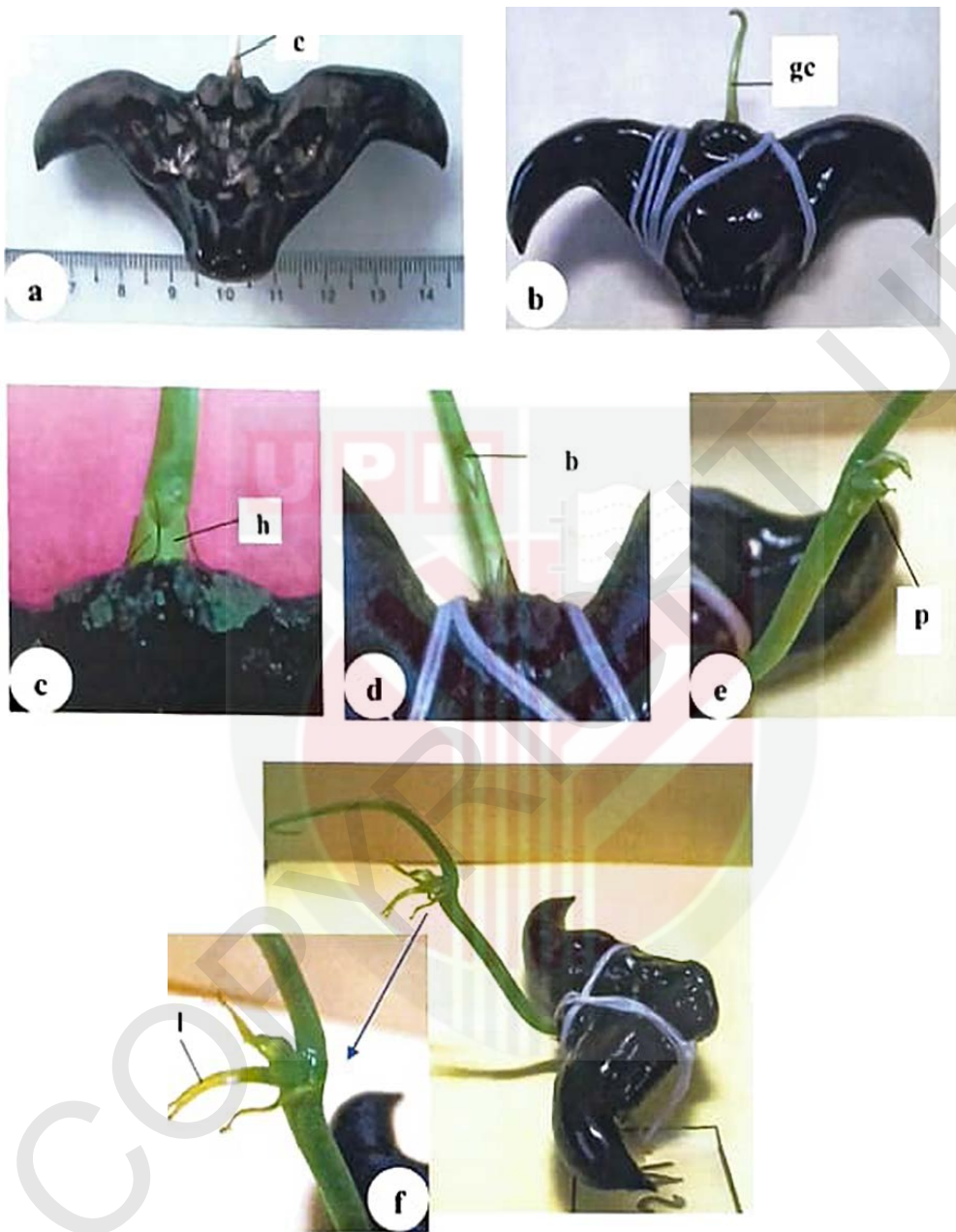


Plate 14: a-Emergence of white cotyledon. b-Green cotyledon, c-Emergence of hypocotyl and d, e. f-Emergence and formation of plumule. c-cotyledon, gc-green cotyledon. h-hypocotyl, b-bulge developed, p-plumule, l-leaves.

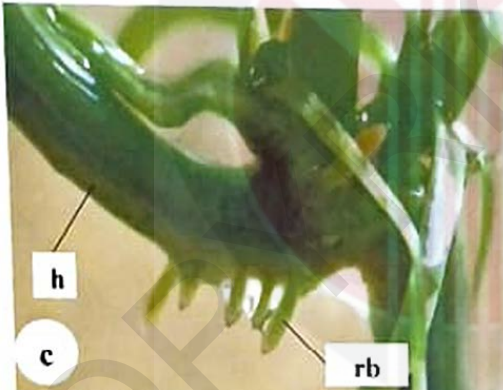
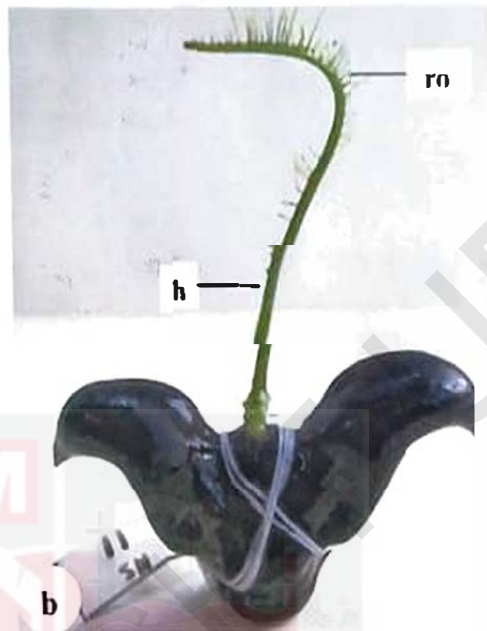
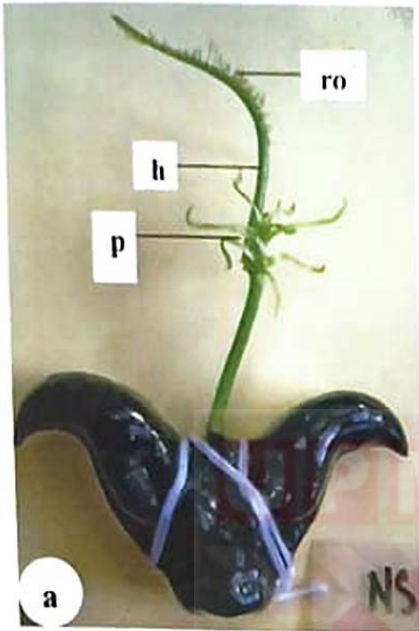


Plate 15: a, b-The formation of roots on hypocotyl and c-The formation of roots below hypocotyls, d- Roots at the plumular site. h- hypocotyl, p-plumule, ro-roots on hypocotyl, rb-roots below cotyledon, rp-roots at the plumular appearance.

The plumule grew into shoot rapidly and form shoot pointing upward (Plate 16a) within 11-40 days. The new leaves also grew in pairs at the internodes (Plate 16a). These leaves are linear, short petioled which represent as juvenile leaves.

The shoot apex also formed linear leaves (Plate 16b) which later form spiral leaves (Plate 16c). These leaves grew broader (Plate 16d) and developed into floating leaves (Plate 16e).

The juvenile leaves at the internodes later abscised and leaved scars at the internodes (Plate 17a). Submerged leaves which resemble like roots simultaneously developed at the scars of the internodes near the bottom (Plate 17a). The submerged leaves were formed in pairs. The roots below hypocotyl were branched and increased in length drastically (Plate 17b). The completely developed young seedling of *Trapa* sp. is shown in (Plate 17c).

The final germination stage of *Trapa* sp. was seed detachment. Seed detachment period is the days for the seed to detach from seedling after germination. The period of seed detachment for seeds imported from Hong Kong (MDS supermarket) ranged from 51-56 days, while the seeds imported from China (Farley supermarket) ranged from 39-59 days (Table 4). The seed detachment period (days) and rate of seed detachment (%) for both seed location shown in Figure 2.

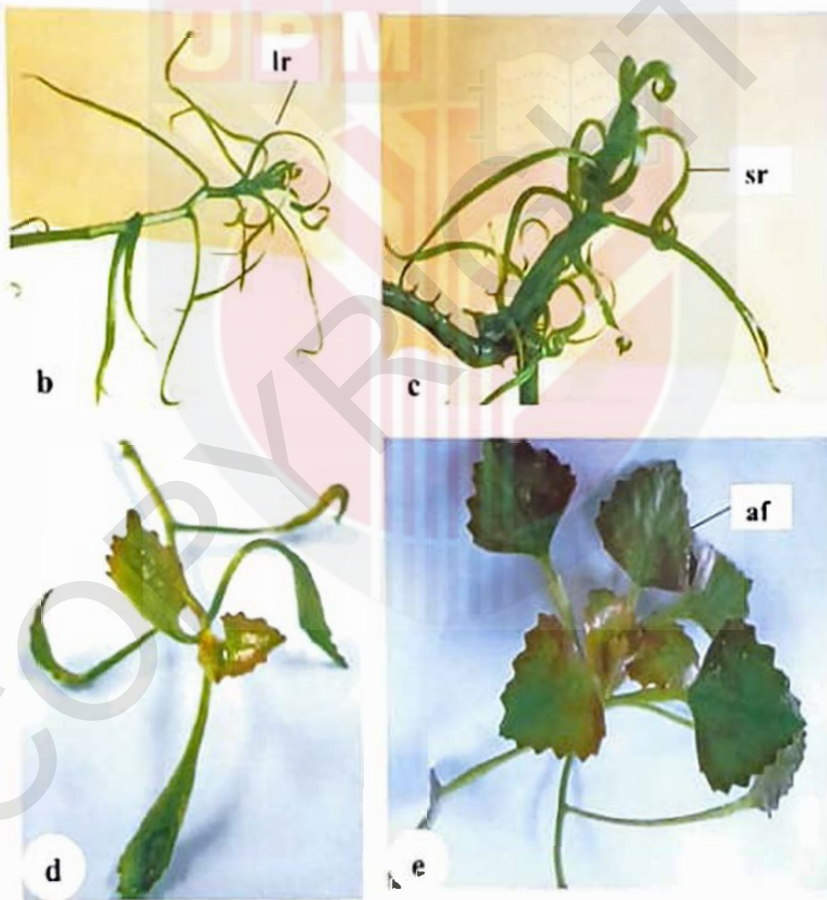
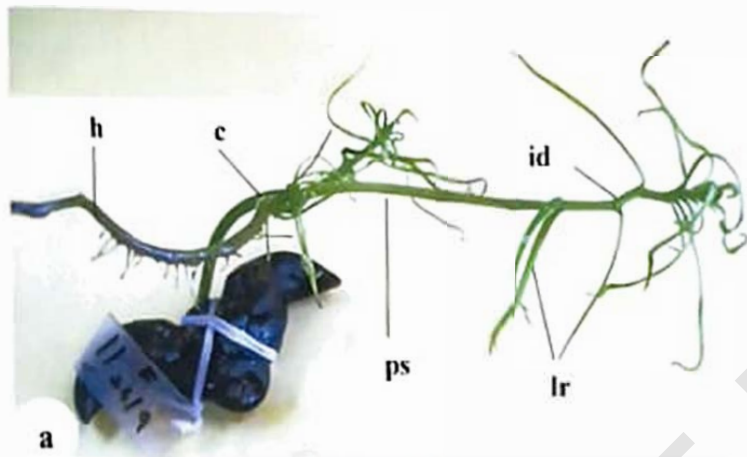


Plate 16: a-Shoot development and formation of linear leaves at the internodes, b-Juvenile phase, linear leaves, c-the spiral form of adult leaves, d-The leaves gets wider and e-The adult floating leaves are formed. h-hypocotyl, c-cotyledon, ps-pioneer shoot, id-internodes, lr-linear leaves, sr-spiral leaves, af-adult floating leaves.

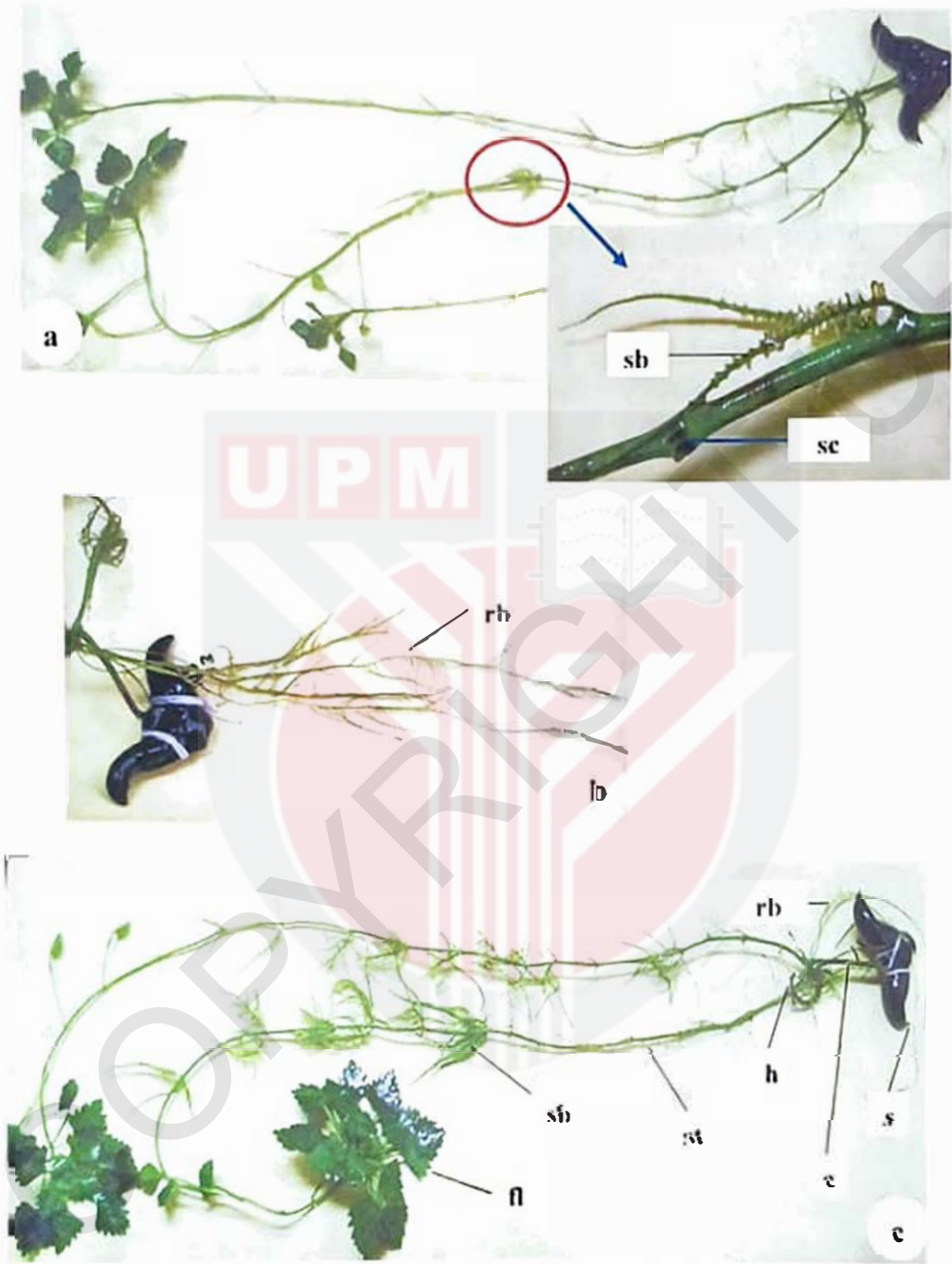


Plate 17: a-Formation of submerged leaves, b- The roots below hypocotyl increased in length and are branched and c- The developed young seedling of *Trapa* sp. sb-submerged leaves, sc-scars at internodes, rh-roots below hypocotyl, fl-floating leaves, st-stem, h-hypocotyl, e-cotyledon, s-seed.

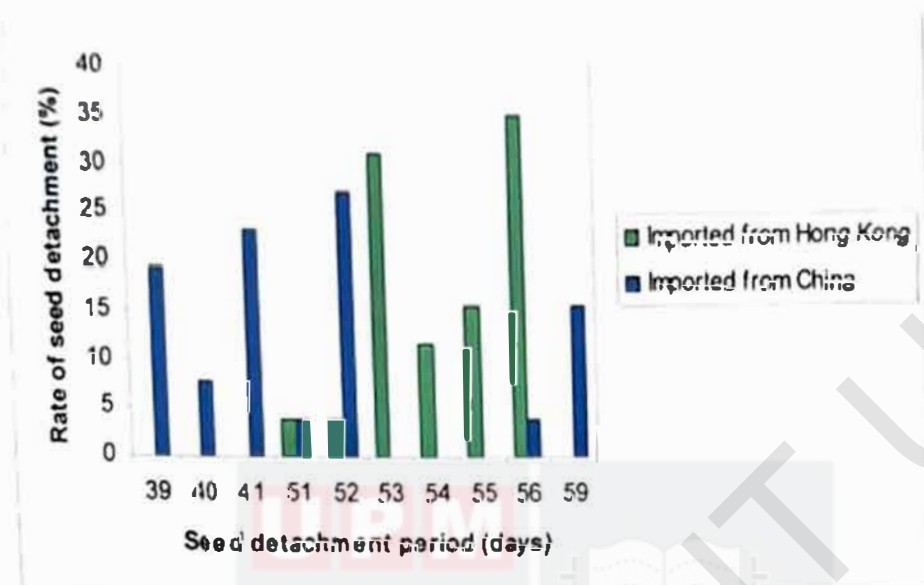


Figure 7: The seed detachment period and seed detachment (%) for both seed sample imported from Hong Kong and China.

4.2.2 Development of mature plant

At young stage the swollen inflated petiole did not formed but at matured period the swollen petiole was very prominent (Plate 18).

The formation of canopy structure was an important event during transition period from young to mature plant. *Trapa* sp produced maximum canopy before the flowering period. New rosettes were developed due to stem branching. The number of rosettes per plant, rosette diameter and number of leaves per rosette from Hong Kong sample are significantly different compared to sample from China (Table 5). However the rate of new leaf emergence per week was similar for both samples, which was 6.93 ± 0.59 for Hong Kong and 6.73 ± 0.46 for China plant (Table 5). As the new leaf emerges, the older moved down the stem. In addition, at this growth period the long unbranched roots at the upper internodes began to develop. The stem and the internodes were also increased in length.



Plate 18: The development of inflated swollen petiole when matured. Scale bar 2cm.

Table 5: Canopy characteristic of *Trapa* sp. through seed propagation.

Seed sample	Hong Kong (MDS supermarket)	China (Farley supermarket)
Parameter	Mean \pm s.d Min – Max 15 samples	Mean \pm s.d Min – Max 15 samples
Number of rosette per plant	2.67 \pm 0.72 ^a (2.00-4.00)	1.93 \pm 0.88 ^b (1.00-4.00)
Rosette diameter (cm)	10.39 \pm 1.12 ^a (8.30-11.5)	11.94 \pm 1.57 ^b (8.90-13.8)
Number of leaves per rosette	21.67 \pm 3.04 ^a (15.00-26.00)	24.00 \pm 1.89 ^b (18.00-25.00)
Leaf emergence rate (leaves per rosette week ⁻¹)	6.93 \pm 0.59 ^a (6.00-7.00)	6.73 \pm 0.46 ^a (6.00-8.00)

Means with same alphabet are not significantly different (t test, $p \leq 0.05$).

4.2.3 Flower development

The flowering stage only achieved by plants from Hong Kong imported seeds (MDS Supermarket). The flowering period of *Trapa* sp. was recorded in the mid month of December. It was almost 3 months from the culture period. During the flowering period one flower per rosette per day is produced. Each flowers blooms only once per day.

In the morning the closed bud emerge on the surface of water (Plate 19a). The petals observed were imbricate because it was enclosed by sepals. As the bud open, the petals expanded slightly (Plate 19b). By afternoon, most buds have opened and the flowers emerged above the water surface (Plate 19c, d). Later at evening, most of the flowers observed had fully opened with petals expanding outwards (Plate 19e). The stamen and stigma were clearly exposed. By late evening most of the flowers began closed with the peduncle curving downward and submerged the flowers.

4.2.4 Fruit development

The fruit development occurs underwater. The fruits matured after one month of flowering. 1-3 fruits per rosette were recorded (Table 6, Plate 20). Floral tube or hypanthium and stigma were found in developing fruits. The mean length of hypanthium was 4.25 ± 1.68 mm and stigma was 5.20 ± 0.17 mm (Table 6). The mean of ovary height and width were 3.35 ± 1.04 mm and 3.12 ± 0.64 mm, respectively (Table 6). The length of developing spine showed huge variation with mean of 12.04 ± 12.04 mm. Meanwhile the mean length of peduncle that holds the developing fruits is 20.76 ± 2.81 mm (Table 6).

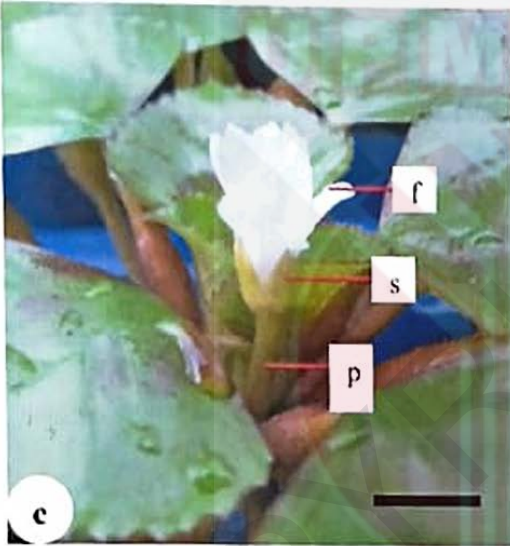
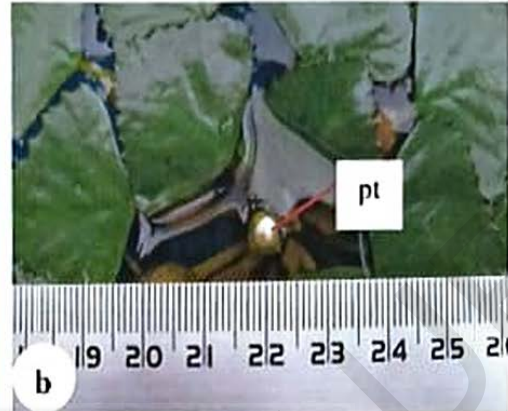
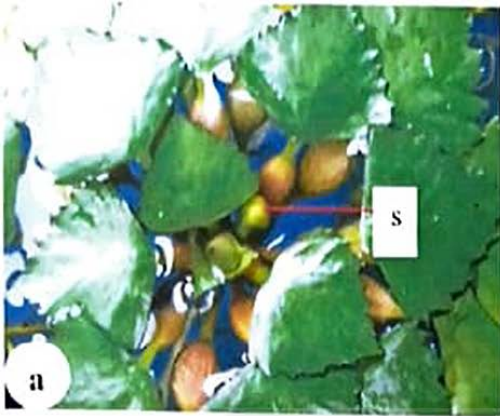


Plate 19: The flowering stage of *Trapa* sp. pt-petal, s-sepal, p-peduncle and f-flower, Scale bar 1 cm.

Table 6: Fruit morphological characteristic of *Trapa* sp. at developing fruit stage.

Parameter	Mean \pm s.d	Min - Max
Number of fruits per rosette	1.70 \pm 0.65 N= 20	1.0-3.0
Hypanthium length (mm)	4.25 \pm 1.68 N= 7	2.50 - 5.97
Stigma length (mm)	5.20 \pm 0.17 N= 7	4.96 - 5.50
Ovary height (mm)	3.35 \pm 1.04 N= 7	2.25- 4.96
Ovary width (mm)	3.12 \pm 0.64 N= 7	2.10-3.96
Developing spine length (mm)	12.04 \pm 12.04 N= 7	2.58-38.70
Peduncle length (mm)	20.76 \pm 2.81 N= 7	15.93-24.98

Data were collected during developing fruit stage. Values denote Mean \pm standard deviation

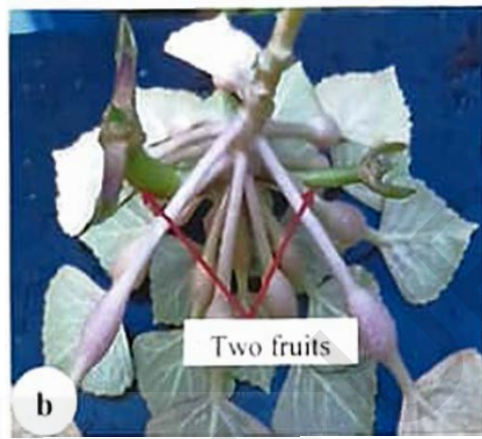
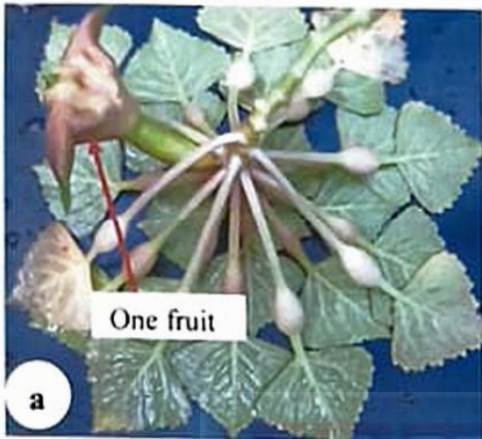


Plate 20: Number of fruits per rosette a-One fruits b-Two developing fruits

At early stage, the calyx was attached to each other (Plate 21a). The stigma was also attached to ovary. Later the calyx or sepals began to expand and pointing outward (Plate 21b, c, d). Throughout this period the ovary increased in length and width (Plate 21e, f). The stigma, petals and hypanthium part is abscised but the calyx remains (Plate 21g). This calyx develops into spines of fruit. Meanwhile the ovary developed into mature fruits.

The fruits ripen during the rainy or cold season. The mature fruits detached from the plant and sink to the bottom of water immediately after ripening and the life cycle of this plant ranged from 3-5 months.

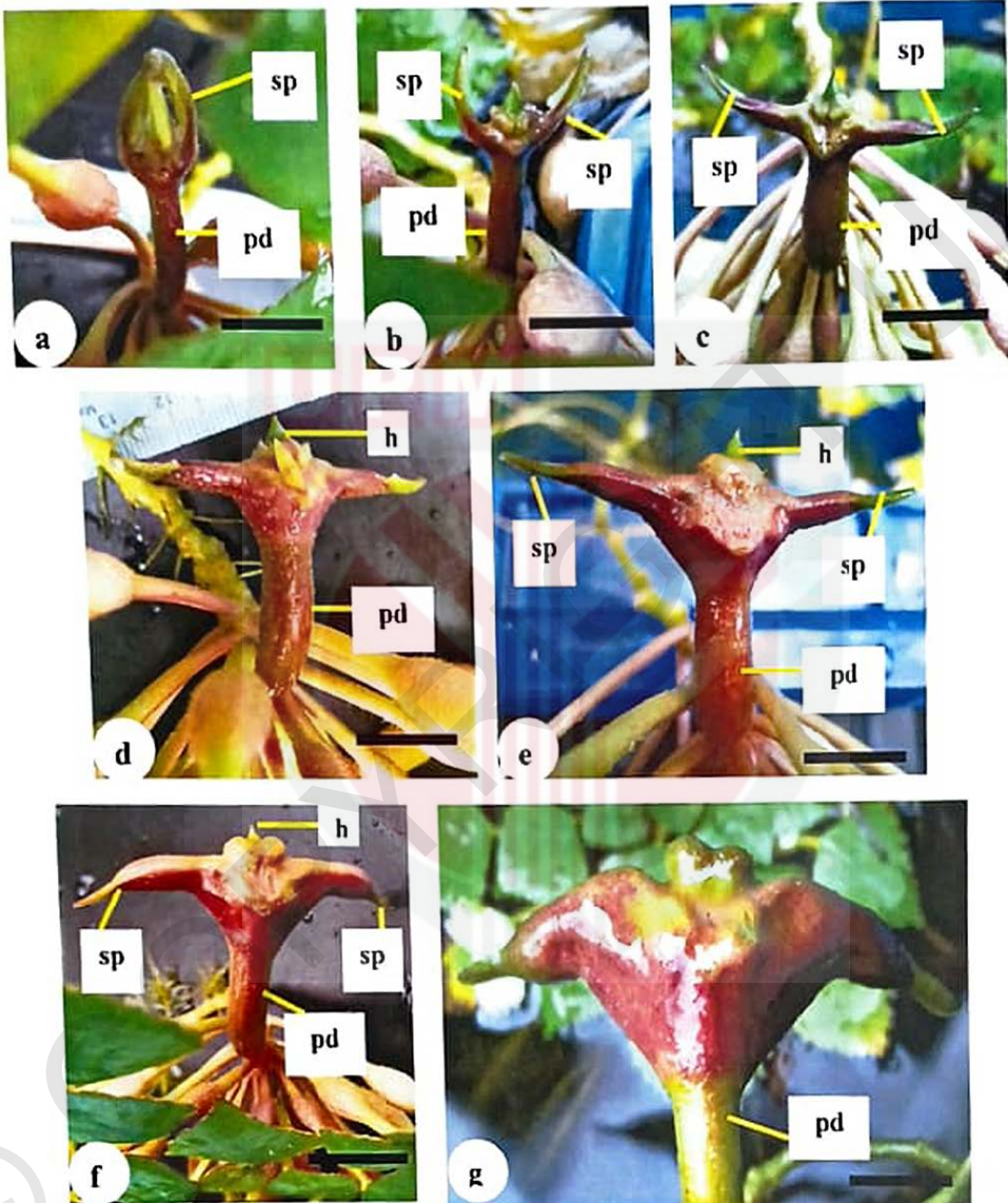


Plate 21: The stages of developing fruits to matured after the pollination process a- Early stage of developing fruit, b-d- Sepals expanding and pointing outward, e-f- Ovary increased in length and width, g- The mature fruit after abscised of hypanthium and the sepals develops into spine. sp- spine, h- hypanthium and pd- peduncle. Scale bar 1cm.

4.3 Stem Cutting Propagation

The growth of *Trapa* sp. by stem or nodal cutting had reached the stage of matured plant. However after transplanting, new rosettes were not developed so the canopy closure was not achieved. The remaining rosettes increased in length and attained a mean maximum length of 8.05 ± 0.02 cm (Table 7). The number of leaves per rosette varies from 12-23 leaves. The leaf emergence per rosette was in the range from 6-7 leaves per week.

The stem elongation was recorded throughout this period. Roots at the upper nodes also developed. Although the plant reached mature stage, the flower and fruit development did not occur. The mature plants did not survive after two months of transplanting.

Table 7: Canopy characteristic of *Trapa* sp. through stem cutting

Parameter	Mean \pm s.d	Min - Max
Rosette diameter (cm)	8.05 ± 0.02	5.8-11.7
Number of leaves per rosette	20.15 ± 1.96	12-23
Leaf emergence rate (leaves per rosette week ⁻¹)	6.03 ± 0.38	6-7

Data were collected from 15 samples when the plants are matured. Values denote Mean \pm standard deviation.

CHAPTER 5

DISCUSSION

In this present study, the vegetative and reproductive morphology of *Trapa* sp. from both sample imported from Hong Kong and China are generally similar. The *Trapa* plants from both locations are identified as *Trapa bispinosa*. The leaves, flower, fruit morphological characteristic and the development of flower and fruit were used to classify the *Trapa* sp. in the present study. The leaf blade measurement, colour of petals, number of horns and the development of *Trapa* sp. from the present study are almost similar to the species of *Trapa bispinosa* Roxb. The detail description of *Trapa* sp. from present study, and others are shown in Table 8.

5.1 Vegetative Morphology

Morphologically, the arrangement of leaves are alternate, forming a terminal rosette, shape of blade are ovate, rhomboid and dentate (Plate 5a) are similar to the description by (Fassett, 1957; Prescott, 1969; Bursche, 1971; Hutchinson, 1973; Cook *et al.*, 1974; Oliver, 1979; Pemberton, 1999; Balgooy, 2001; Hummel and Kiviat, 2004; Charles and O'Neill, 2006). The leaf blade are bright green in colour, upper surface is glossy, prominent veins below surface. Hummel and Kiviat (2004) reported the colour of below surface of floating leaves are yellowish green, however in this study are pale green in colour (Plate 4c).

Table 8: The morphological and development description of *Trapa* sp.

Species	Leaf Morphological Characteristic of <i>Trapa</i> sp.						Submerged leaves
	Floating leaves						
	Colour of leaf blade	Shape of leaf blade	Measurement of leaf blade	Petiole	Other characteristic	Characteristic	
<i>Trapa</i> sp. (Present study from Hong Kong and China imported plants)	Matured leaf is green and young leaf is reddish green in colour, the lower surface of matured and young leaves are pale green in colour	Leaf blade ovate in outline, rhomboid and dentate with sharply serrated leaf margin finely divided into two, leaf tip is acute and not serrated or divided	Leaf blade from Hong Kong sample 24.36-32.39 x 27.87-38.42 mm	Inflated swollen petiole at above the middle, hairy Petiole length from Hong Kong is 31.01-62.25 mm Petiole length from China is 31.09-50.17 mm	Glossy upper surface and below surface are leathery with prominent veins, floating leaves are terminal in rosette, whorled structure with alternate arrangement	1-2 pairs, green in colour, branched resemble like-roots	
			Leaf blade from China sample 11.36-20.14 x 12.28-24.50 mm				
<i>Trapa bispinosa</i> Roxb. (Source from: Flora of Pakistan, online www.efloras.org .)	-	Dentate to crenate at the apical end	Leaf blade 2.5 x 3.5-7.0 cm	Petiole 3-12 cm long, woolly, fusiform, swelling at the upper end	Glabrous above the leaf blade and densely pubescent-villous below leaf blade	Present	
<i>Trapa bispinosa</i> Roxb. (Oliver, 1979)	-	-	Leaf blade varies to 3 inches in width	Petiole is hairy	Tomentose/Leathery beneath the leaves	Present	
<i>Trapa bispinosa</i> (Subryamanyam, 1962)	-	Leaf blade rhomboid	-	Swollen petiole present	Leaves very villous beneath, form rosulate leaves	Free-floating, borne in pairs, branched, green	

<i>Trapa bicornis</i> var. <i>cochinchinensis</i> (Pancho and Soerjani, 1978)	Green in colour	-	Leaf blade 5-7 x 6-9 cm	Petiole 9-20 cm long	-	-
<i>Trapa maximowiczii</i> (Pancho and Soerjani, 1978)	Toward the base black-brown or with two dark spots	Leaf blade dentate	Leaf blade 2.5-4.0 x 3.5-6.0 cm	Petiole 5-15 cm long, bulbous in the upper half	-	-
<i>Trapa quadrispinosa</i> Roxb. (Source from: Flora of Pakistan, online www.efloras.org)	-	Leaf blade dentate	Leaf blade 1-3 x 1.5-4 cm	Petiole up to 10 cm long, dilated near the leaf base villous	The leaves are glabrous above, pubescent villous below especially on the nerves	Present
<i>Trapa incisa</i> (Jiarui et al., 2000)	Glossy and dark green adaxially and purplish abaxially, with black brown or 2 dark spots	Leaf blade rhomboid and triangular, the leaf margin coarsely sharply incised, dentate distally	Leaf blade 1.5-3 x 2-4 cm	Petiole 5-15 cm, slender, slightly swollen or not	The leaf blade glabrous/ sparsely pubescent on veins	Present and mistaken as adventitious roots
<i>Trapa natans</i> (Jiarui et al., 2000)	Glossy and dark green adaxially, greenish purple abaxially, with coloured spots between veins	Leaf blade shape varies from deltoid to rhomboid, the leaf margin is dentate distally	Leaf blade 4-6 x 4-8 cm	Petiole 5-18 cm, swollen distally, pubescent	Glabrous at the upper surface of leaves	Present and mistaken as adventitious roots
<i>Trapa natans</i> (Oliver, 1979)		Leaf blade base broadly cuneate, outer margin dentate	Leaves varies 1-2 inches in width	Petiole glabrescent	Leaf blade is glabrous above, glabrescent or papillose	-

Species	Flower and Fruit Development and Morphological Characteristic of <i>Trapa</i> sp.		
	Flower	Fruit	
	Characteristic	Number of horns and shape of spine	Other characteristic of fruit
<i>Trapa</i> sp. (Present study from China and Hong Kong)	Hermaphrodite, solitary in leaf axils, the peduncle is hairy, four sepals, four white petals, four yellow stamens and one rounded stigma.	2 horns, sharp and blunt spine	The fruit is indehcent, 5.12-25.60 x 3.40-23.90 mm. The spine length varies from 16.19-66.43 mm
	Flower and fruit development of <i>Trapa</i> sp.		
	The flower blooms fully and emerged at the surface of water in the afternoon, after pollination peduncle curved bend down and submerged the flowers. The fruit formation and development occurs underwater during rainy and cold season		
<i>Trapa bispinosa</i> Roxb. (Source from: Flora of Pakistan, online www.efloras.org .)	White, villous, sepals ovate, Petals ablong, narrow base twice length of sepals, stigma rounded	2 horns pointing upwards	Broad, quadrangular, top shaped, barbed below the tip of fruit Fruit is 2.0-3.8 cm long and broad
<i>Trapa bispinosa</i> Roxb. (Oliver, 1979)	-	2 horns, slender patent lateral horns, the intermediate teeth undeveloped	Apex shortly apiculate with persistent style base
<i>Trapa bispinosa</i> (Subryamanyam, 1962)	White in colour	2 horns, upwardly projecting spine	-
	Flower and fruit development of <i>Trapa</i> sp.		
	The flower open above the surface of water in the afternoon, after pollination the pedicel bends down so that the flower is submerged and the fruit formation take place at this condition, flowering happens during rainy season and the fruits ripen in the cold season.		

<i>Trapa bicornis</i> var. <i>cochinchinensis</i> (Pancho and Soerjani, 1978)	-	2 horns, blunt, straight or subcurved horns	-
<i>Trapa maximowiczii</i> (Pancho and Soerjani, 1978)	Petals are pale lilac, anthers yellow	4 horns, horns often unequal, straight, or curved	Retrorsely barbed spine
<i>Trapa guadrispinosa</i> Roxb. (Source from: Flora of Pakistan, online www.efloras.org)	Sepals ovate, especially in the middle portion, petals 8 mm and white	4 horns with two are shorter from the other two horns	Fruit 1.3x 1.7 cm.
<i>Trapa incisa</i> (Jiarui <i>et al.</i> , 2000)	Petals pink to pale purplish or white, 5-7 mm	4 horns, lower horns descending and upper horns horizontal to ascending	Fruit narrowly rhomboid, the surface variously ribbed to smooth, beak finely conic, apex barbellate Fruit 0.8-1.5 x 1.2-2 and thickness of the fruit is 0.7-1 cm
<i>Trapa natans</i> (Jiarui <i>et al.</i> , 2000)	Petals white, 7-10 mm	2-4 horns, horns horizontal to ascending, recurved	Crest a prominent bulge to a thin rib, , beak conic with turf of hairs Fruit is 1.8-3 x 2-4.5 and thickness of the fruit is 1-2.8 cm
<i>Trapa natans</i> (Oliver, 1979)	-	4 horns, short broad based horns	-

The leaves were also leathery at below surface as discussed by Hummel and Kiviat (2004), Oliver (1979) and sharply serrated (Fassett, 1957; Cook *et al.*, 1974 and Hummel and Kiviat, 2004). However only the edges of leaves margins were serrated and finely divided into two and the leaf tip is not serrated (Plate 5b, c, d). The swelling and spongy inflated petiole observed were above the middle of petiole (Plate 6a) In contrast Jiarui *et al.*, 2001 stated the petiole is often about the middle. The hairy petioles were determined (Oliver, 1979).

The floating leaves comprises of leaf blade, petiole and inflated petiole. The measurement on floating blade length and width for Hong Kong (MDS supermarket) plant is 28.38 ± 2.04 and 31.30 ± 2.04 mm while China (Farley supermarket) plants are 18.47 ± 2.78 and 20.45 ± 3.18 . This showed that blades are almost as broad as the long which was mentioned by Fassett (1957). Oliver (1979) mentioned that the leaf blades width varies nearly to 3 inch. The petiole length for Hong Kong (MDS supermarket) and China (Farley supermarket) sample are 50.27 ± 7.33 and 42.99 ± 5.46 respectively. The petiole width showed a distinct difference compare to inflated petiole due to the swelling feature of inflated petiole.

The submerged leaves formed at the internodes after abscission of young leaves, green and opposite arrangement agree with many authors (Nicholson and Evelyn, 1969; Hutchinson, 1973; Cook *et al.*, 1974; Agarawal and Mohan Ram, 1995; Jiarui *et al.*, 2000; Balgooy, 2001; Hummel and Kiviat, 2004). However, these submerged leaves were mistaken as adventitious roots (Prescott, 1969; Cook *et al.*, 1974; Agarawal and Mohan Ram, 1995; Jiarui *et al.*, 2000) due to the root like structure. The green colour

and lack of root characteristic supported that it was submerged leaves. The leaves were also observed in 1-2 pairs.

The stem is long, slender, submerged and bear the fibrous roots are similar to Jiarui *et al.*, (2000). The width of stem for Hong Kong (MDS supermarket) and China (Farley supermarket) sample ranged from 1.81-2.30 mm and 1.94-2.15 mm respectively. According to Jiarui *et al.*, (2000) the stem diameter were stated as 2.5-6.0 mm. The colour of green stem during young and changed to brown when matured. At the lower part of stem the branched, green, fibrous roots are formed and identified as anchoring roots (Plate 10b). However the anchoring roots were described as branched (Ahmad and Singh, 1998) and white in colour (Agarawal and Mohan Ram, 1995). The reddish brown floating roots were unbranched and were found at the upper internodes when matured (Plate 10d).

5.1 Reproductive morphology

The flowers of *Trapa* is white in colour, solitary peduncle in leaf axils and comprises 4 sepals, 4 white petals, 4 yellow stamens, one stigma, and also have an ovary half-inferior as similarly reported by Nicholson and Evelyn (1969), Hutchinson (1973), Cook (1974), Cook *et al.*, (1974), Oliver (1979), Ahmad and Singh (1998), Pemberton (1999), Jiarui *et al.*, (2000), Balgooy (2001) and Hummel and Kiviat (2004). The flower is hermaphrodite having male and female structure (Cook *et al.*, 1974; Jiarui *et al.*, 2000; Balgooy, 2001). The peduncles of flowers were hairy and can be observed with naked eyes.

The fruit is indehiscent with two horns, triangular, hard shell and black in colour are similarly described by Nicholson and Evelyn (1969), Hutchinson (1973), Ridley (1980), Benson (1979) and Hummel and Kiviat (2004). The fruits apex was barbellate which was described by Jiarui *et al.*, (2000). Huge morphological variations in dimension for the fruit body are expressed as fruit height, width, spine length and peduncle length for attached fruits. This showed the fruits varied from each other greatly. The blunt and sharp spines were also identified (Suriyagoda *et al.*, 2006, 2007).

The seed of *Trapa* has two unequal white cotyledons as the small and large one (Hutchinson, 1973; Cook, 1974; Agarawal and Mohan Ram, 1995). The seed is also non-endospermic (Hutchinson, 1973; Cook, 1974; Agarawal and Mohan Ram, 1995).

5.3 Vegetative and Reproductive Propagation

5.3.1 Seed germination

The percentage germination of imported seeds from Hong Kong showed almost 50% higher compared to imported seed from China. The ungerminated seeds are due to the severity of the germination pit injury (Kurihara and Ikushima, 1991). Beside that some of the seeds were identified in dormant stage. The dormant and newly produced seeds are distinguished by existence or absence of pericarp (Kurihara and Ikushima, 1991; Ahmad and Singh, 1998). *Trapa* sp. is classified as recalcitrant seeds and as the other aquatic plant it exhibit dormancy due to hard seed coat (Agarawal and Mohan Ram, 1995; King and Roberts; 1980).

The seed germination procedure was done under room temperature which was about 26°C-28°C. According to Sharma *et al.*, (1984) high temperature or acid scarification treatment may increase the germination but Kurihara and Ikushima (1991) reported that, seeds of *Trapa* sp. germinated more synchronously in a shorter period at high temperatures in the laboratory but the temperature factor did not influence the final or maximum rate of germination.

The germination period and rate of germination for both samples were not consistent as in Figure 1. The variation in germination period may due to the need for ripening period of the embryo and the presence of germination inhibitors which need to leach out before germination (Birch, 1981).

Germination Stages

Seven germination stages of *Trapa* sp. were recognized and the range for both seed sample from Hong Kong and China took to 39-59 days to complete the stages (Table 4).

The white cotyledon emerged and turn to green in 1-2 days while Agarawal and Mohan Ram (1995) reported it remain quiescent for 4 weeks before it turn to green using *in vitro* techniques. The radicle was absent and no primary roots developed (Agarawal and Mohan Ram, 1995). However, Hutchinson (1973) and Oliver (1979) described the radicle germinated together with the small cotyledon on top of the fruit.

The emergence of hypocotyl and plumule was similar as discussed by Agrawal and Mohan Ram (1995). The hypocotyl region is known can elongate carrying with it the

node which the plumule and leaves developed (Birch, 1981). The first leaves emerged were identified from the plumule (Plate 19). The formation of roots along the hypocotyl axis occurred after the emergence of plumule. In contrast Agarawal and Mohan Ram (1995) explained the roots on hypocotyl were initiated before plumule development.

The anchoring roots below the hypocotyl were present and observed (Plate 21) but the roots were not formed under *in vitro* condition (Agrawal and Mohan Ram, 1995). The stage of developing shoots and young seedling which involves the formation of linear leaves at the internodes, transition from linear, spiral to adult floating leaves and formation of submerged leaves were similar to Agarawal and Mohan Ram (1995).

The range of days taken for both seed samples to achieve the germination sequence showed less variation except for the seed detachment period from the seedling. The detachment period and rate of detachment (%) for both seed sample varies as shown in Figure 2. This indicated the seeds attach to the seed to utilize the food resource supplied by the embryo. This is supported by Kunii (1988) who reported that successful seedling establishment is the possession of a large seed which provides ample reserve of nutrients during the period immediately after germination. So as the seedling finish had finish use the food reserves, the seed detached. The various seed sizes also related to utilization of reserve nutrient, as the smaller seeds will detach earlier than the larger one. After the seed detachment the juvenile plant required substrate and nutrient for growth.

5.3.2 Development of mature plant under vegetative and reproductive propagation

The seed germination and stem cutting undergo the transition process from young seedling to mature plant. Swollen petioles were developed for both propagation methods. Rosette number is an important component because the rosettes bear the fruit. The new rosettes are produced from the lateral bud on the stem (Arima *et al.*, 2006; Suriyagoda *et al.*, 2006, 2007). New rosettes were found in the plant through seed propagation but none for stem cutting plants. The rosette diameter and number of leaves per rosette in seed propagation showed more productive compared to stem cutting. However the leaf emergence rate per week was almost same for both methods (Suriyagoda *et al.*, 2007). Furthermore, Kunii (1998) reported the suggested constant birth and death of individual leaves was approximately one leaf per day and estimated the life span of *Trapa* sp. to be less than 30 days.

5.3.3 Flower and fruit development under vegetative and reproductive propagation

The *Trapa* sp. plant derived from seed propagation reached the flowering to fruiting development stages. The floral sequences of events leading to and during anthesis were same as discussed by Kanodo (1986). Furthermore, the flower bud formation of water chestnut was found to be under control of day length (Suriyagoda *et al.*, 2007). Kanodo (1986) reported due to the stigma and anther position, the insect movement within the flower caused the anther sacs being pushed against stigma facilitating self pollination.

The flowering to fruiting duration period recorded were almost one month. The process of developing spines were same as reported by Kanodo (1986) where the stigma, petals and hypanthium part is abscised but the calyx persist to develop spines (Plate 21). The fruits after mature will detach and sink to the bottom due to the heaviness of seed (Kunii, 1988). The life cycle of the plant completed in 3-5 months period.

The vegetative propagation method using stem cutting plants did not achieved the flowering and fruiting stages as recorded in seed propagation. In addition Agarawal and Ram (1995) had outlined that *Trupu* sp. exhibits a strong apical dominance and lack a natural means of vegetative propagation. The life span of the plant was only about 2 months.

CHAPTER 6

CONCLUSION

The vegetative and reproductive morphology of *Trapa* sp. were observed in this study from Hong Kong (MDS supermarket) and China (Farley supermarket) sample. Seed germination and vegetative propagation using stem cutting were determined. The morphology characteristic description from both locations agrees with many authors (Fassett, 1957; Subramanyam, 1962; Prescott, 1969; Bursche, 1971; Hutchinson, 1973; Cook *et al.*, 1974; Pancho and Soerjani, 1978; Pemberton, 1999; Balgooy, 2001; Hummel and Kiviat, 2004; Charles and O'Neill, 2006) and the *Trapa* plants from both locations are identified as *Trapa bispinosa* Roxb.

Based on the leaf morphology characteristic, it comprises two kinds of leaves which are the floating and submerged leaves. The floating leaves are alternate, crowded terminally into a rosette, hairy petiolate and inflated petiole above the middle. The leaf blade are bright green colour, prominent veins and leathery below surface, serrated at the edges of leaf margin but not at the tip of leaf. The measurement for blade length, blade width, petiole length, petiole width, inflated petiole length and inflated petiole width for both locations from Hong Kong (MDS supermarket) and China (Farley supermarket) varies from each other. The submerged leaves are opposite, 1-2 pairs, resemble like roots and formed after the juvenile leaves abscised at the scars of internodes. The stem is long and submerged. Two types of roots were also determined. The anchoring roots formed first and followed by the reddish-brown at the upper part of internodes.

The flowers of *Trapa* sp are hemaphrodite, emerged at the leaf axils of floating leaves and hold by hairy peduncle. The flowers are white in colour with 4 petals, 4 stamens and one stigma. The fruits are indehiscent with 2 horns, black in colour and hard, apex harbellate with great morphological variation in the fruit height, fruit width and spine length. The seeds are unequal in size where the large one retained in fruit and the small one germinated from the fruit apex.

Germination percentage for Hong Kong (MDS supermarket) imported seed sample are higher compared to the China imported seeds (Farley supermarket) due to the seed condition. Seven germination stages are identified which are emergence of cotyledon, emergence of hypocotyl, emergence of plumule, formation of roots on hypocotyl, formation of roots below hypocotyl, developing shoots and young seedling and seed detachment. The seeds from both locations took 39-59 days to accomplish the seven germination stages.

The young seedling reached the mature plant stages and the canopy formation is an important characteristic at this stage. The flowering and fruiting development were only observed in the Hong Kong (MDS supermarket) sample. The life cycle of *Trapa* sp for this sample ranged from 3-5 months. The *Trapa* sp. plant using stem cutting reached the matured stage, however the flowering and fruiting stages were not determined. The lifecycle was only about 2 months. The seed propagation showed a better establishment compared to the stem cutting propagation. The fruit characteristic have the biotechnology and economic importance, thus crop management and improvement studies would open a new way in production of water chestnut.

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PUBLICATION OF THE PROJECT UNDERTAKING

This is to certify that I have no objection to publish the project entitled "Vegetative and Reproductive Propagation of Water Chestnut, *Tropa sp.*" by the supervisor in a joint authorship. However, it has to be evaluated by the Faculty of Agriculture and Food Sciences, Universiti Putra Malaysia Bintulu Campus and published in the form approved by the Faculty.



Menaka D/O Ganeson

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