CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION OF *Nelumbo nucifera* Gaertn.

Nelumbo nucifera Gaertn. or commonly known as ''lotus'' is a perennial and horizontal aquatic emergent angiosperm plant, large and rhizomatous aquatic herb with slender, elongated, branched, creeping dimorphic stem (consisting of nodal roots); leaves are membranous, net vein, peltate (60-90 cm and above), orbicular and concave to cupshaped; petioles are long (up to 3 m), rough with small distinct prickles, contains milky latex; inflorescence on an elongate, axillary, spineless pedicel; flowers are pink and white, sweet-scented, solitary, hermaphrodite (bisexual) ,large (10-25 cm diameter); ripe carpels are 12 mm long, ovoid and glabrous; fruits are ovoid having nut like achenes; seeds are dicotyledonous, large, black, hard and ovoid. Dispersal is by seed or rhizome fragments. Seed longevity up to several hundred years.

Lotus is introduced as fresh water ornamental plant or an edible vegetable in numerous countries around the world categorised as one of the endangered aquatic plants and only two species remains, *Nelumbo nucifera* Gaertn. (Figure 1.1) and *Nelumbo lutea* (Willd) Pers. (Figure 1.2).

Figure 1.1 : *Nelumbo nucifera* Gaertn. (sacred lotus or pink Asian lotus) in Chini Lake, Pahang, Malaysia.

Figure 1.2 : *Nelumbo lutea* (Willd) Pers. (yellow American lotus) in North America, United States of America.

Lotus is a typical hydrophyte that has been cultivated to produce edible tubers in the wetlands of eastern Asian countries. This species has a large, round leaf that expands horizontally at the top of its petiole. The diameter and area of the leaf exceed 0.5 m and 0.25 m, respectively, in a full growing period (Takagi et al., 2006).

There is a common red-flowered form where petals have darker red lines and a very rare form where the petals are dark red at the apices; less common are a white form and an intermediate white-red form. Flowers with less than 25 petals are usually known as singled-flowered, those with 25-50 petals are known as intermediate and those with over 50 petals are called double-flowered (La-ongsri et al., 2008).

The lotus is an important aquatic economic plant, not only as a dainty and ornamental flower but also as vegetable with the source of herbal medicine and strong bioactive ingredients such as alkaloids, flavonoids, antioxidants, antisteroids, antipyretic, anticancerous, antiviral and anti obesity properties (Mukherjee et al., 1997; Sinha et al., 2000; Qian, 2002; Sridhar and Bhat, 2007).

Lotus as one of most important aquatic plants has critical role in ecological systems of wetlands, lakes and ponds. The growth of lotus is also affected by water level and its fluctuation. The deepest water level recorded is about 2-3 m for wild lotus (Wang and Zhang, 2005). Although, lotus is an aquatic plants, flooding during growing season is a critical factor for surviving. However, some emergent plants have characteristics to adap the water level fluctuations (Vergara, 1985; Blom et al, 1990).

Aquatic vegetable production also can resolve the problem of water eutrophication due to high biomass and high rate of harvest of crops such as arrowhead, water bamboo, lotus root, water dropwort (Wang, 2007). Recently lotus has been included in the list of endangered species in China (Dong and Zheng, 2005) and America (Sayre, 2004).

Aquatic ecosystems such as rivers, lakes, ponds and wetlands, are expected to be the most severely affected by global warming phenomenon such as UVB radiation, decreasing water quality, salinisation, elevated temperature and water level as they occupy the lowest areas in the landscape (Brock et al., 2005; Goodman et al., 2010; Hart et al., 2003; James et al., 2003; Walker et al., 2002).

Despite being recognised as areas of ecological complexity and conservation importance (Davis et al., 2006), aquatic systems continue to be among the world's most threatened ecosystems (Zedler and Kercher, 2005). The threats will lead the aquatic biota become increasingly stressed, resulting in reduced growth and reproduction (Sim et al., 2006) and ultimately death (Kefford et al., 2007; Nielsen et al., 2003), leading to a decline in species richness (Hart et al., 1990).

During the past few years, there has been increasing interest in the use of aquatic vascular plants for the removal of pollutants from domestic and industrial sewage effluents (McDonald and Wolverton, 1976).

Lotus is a fertilizer-consuming plant and live in a soil-aquatic environment. Currently, organic fertilizer is stil a major source of fertilizer in China and manufactured N-P-K fertilizers are widely used in Japan for production of vegetable lotus (Sou and Fujishige, 1995). At the same time, production of ornamental lotus in containers and using soluble fertilizer (tablet) at the nursery becoming a potential plant in horticultural industry (Creamer, 2008) and nursery production.

Nelumbo is a good bioindicator of trophic changes, assimilation and removal of heavy metals. The ability of *Nelumbo nucifera* in chromium removal and in bioassay was assessed (Vajpayee et al., 1999). Lotus can absorb and accumulate the heavy metal elements including Fe, Mn, Cu, Zn, Pb, and Ca (Sun et al., 1996).

Nelumbo nucifera (lotus), is widely grown as an economical crop in Asian countries including China, Japan, India, Korea, Nepal, Thailand, Vietnam, Sri Lanka, Philippines and Indonesia. In Thailand, lotus is considered as a source of three of four necessities including life, food, medicine and clothing (Chomachalow, 2007). Nutritious parts of lotus rhizomes containing alkaloid and liensinine are highly demanded as vegetable in Japan, South Korean and Asian (Zhao, 1999).

China is the largest country in producing both seeds and rhizomes of lotus and distributed widely in of south China, especially in Yellow River where water, heat and light are plentiful. The growing area of lotus in China is estimated to be 0.3 million ha (Li et al., 2005a). Japan produced 82200 tons of lotus rhizomes in 1982 on an area of 6350 ha (Nguyen, 2001). Recently, lotus is becoming a potential crop in Australia

(Nguyen,2001), New Zealand (Follett and Douglas, 2003) and United States (Tian et al., 2006).

Lotus, one of the 26 vegetables with largest sale in China (Jiang and Cao, 2005), is also an important export for their potential as a vegetable and ornamental water plant. All the products were export to Japan, South Korean and Asian (Zhao, 1999). In 2002, lotus exported has been estimated to be more than 0.1 million tons to Baoying County, Jiangsu province. Meanwhile, 75% was exported to Japan (Li et al., 2003). Lotus has been cultivated as an ornamental and food plant in Japan for more than 1000 years (Masuda et al., 2006).

Today, hundreds of *Nelumbo* cultivars were commercialized, mostly in Asia (Zou et al., 1997) through hybridization for ornamental and vegetable purposes. According to Guo (2008), up to the year 2002, a total of 572 lotus accesions (including landraces, cultivars and breeding lines) were conserved in National Garden of Aquatic Vegetable, Wuhan, Hubei province as the concern of this nutritiously endangered aquatic plants (Figure 1.3 and Figure 1.4).

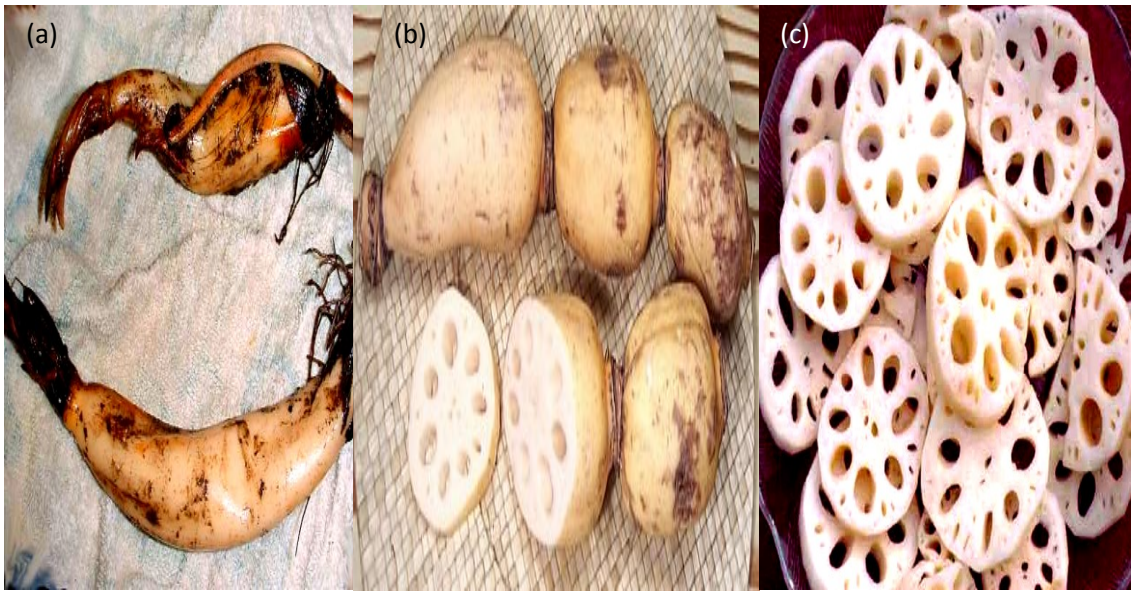


Figure 1.3 : Nutritious parts of lotus rhizomes containing alkaloid and liensinine (a) rhizome (b) nodal buds (c) edible lotus rhizome.

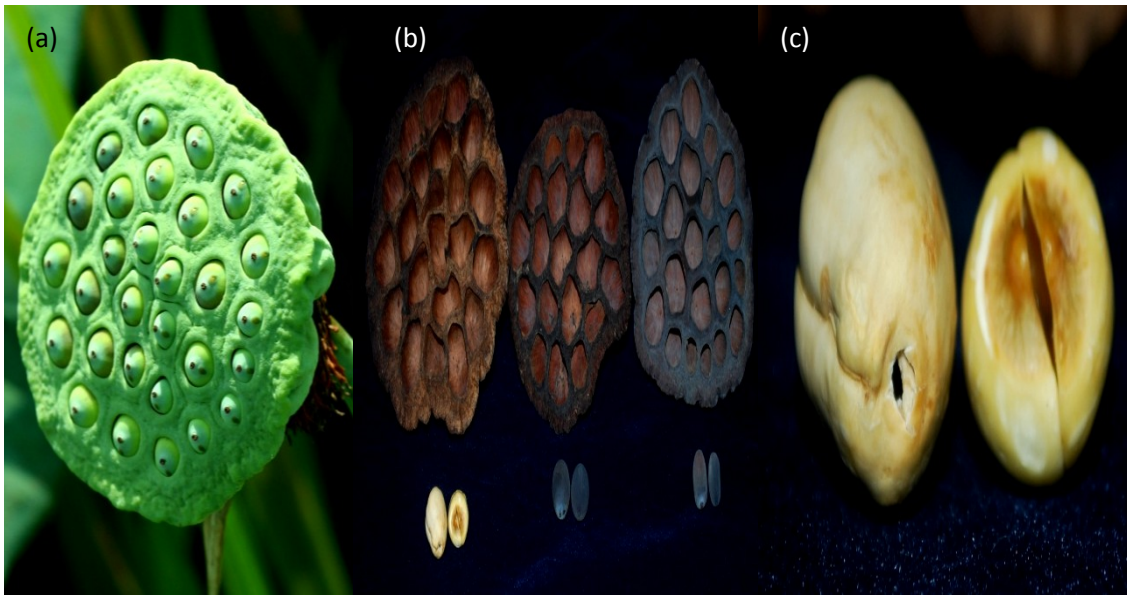


Figure 1.4: Nutrious parts of lotus fruits containing containing alkaloid, liensinine, saponins, phenolics and carbohydrates (a) green fruit (b) matured seeds (c) dried lotus nut.

1.1.1 Classification and Nomenclature of *Nelumbo* sp.

- Kingdom : Plantae
  - Division : Magnoliophyta (Angiospermae -Flowering plants)
    - Class : Magnoliopsida (Dicotyledons)
      - Subclass : Magnoliidae
        - Order : Nymphaeales
          - Family : Nelumbonaceae (Lotus lily)
            - Genus : *Nelumbo*
            - Species :
              - i) *Nelumbo nucifera* Gaertn.
              - ii) *Nelumbo lutea* (Willd.) Pers.

1.1.2 Comparison of Characteristics of Nelumbonaceaea (lotuslily) and Nymphaeae (waterlily)

The genus *Nelumbo*, commonly known as lotus, was previously included in the family Nymphaeaceae. However, based on recent molecular phylogenetic analyses **(**Barkman et al., 2000; Lee and Wen, 2004), many researchers now place the genus in a separate family, Nelumbonaceae (Kubo et al., 2009). The former Nelumbonaceae is distributed in Asia and North Australia and the latter is found in North and South America (Borsch and Barthlott, 1994; Han et al., 2007).

Lotus and water lily are frequently confused but the plants are actually have their own characteristics that can be differentiated. However, many reasons for the recent assignment of the *Nelumbo* genus into its own separate family, the Nelumbonaceae. The physiological differences between Nelumbonaceae (lotuslily) and Nymphaeae (waterlily) were listed in Table 1.1.

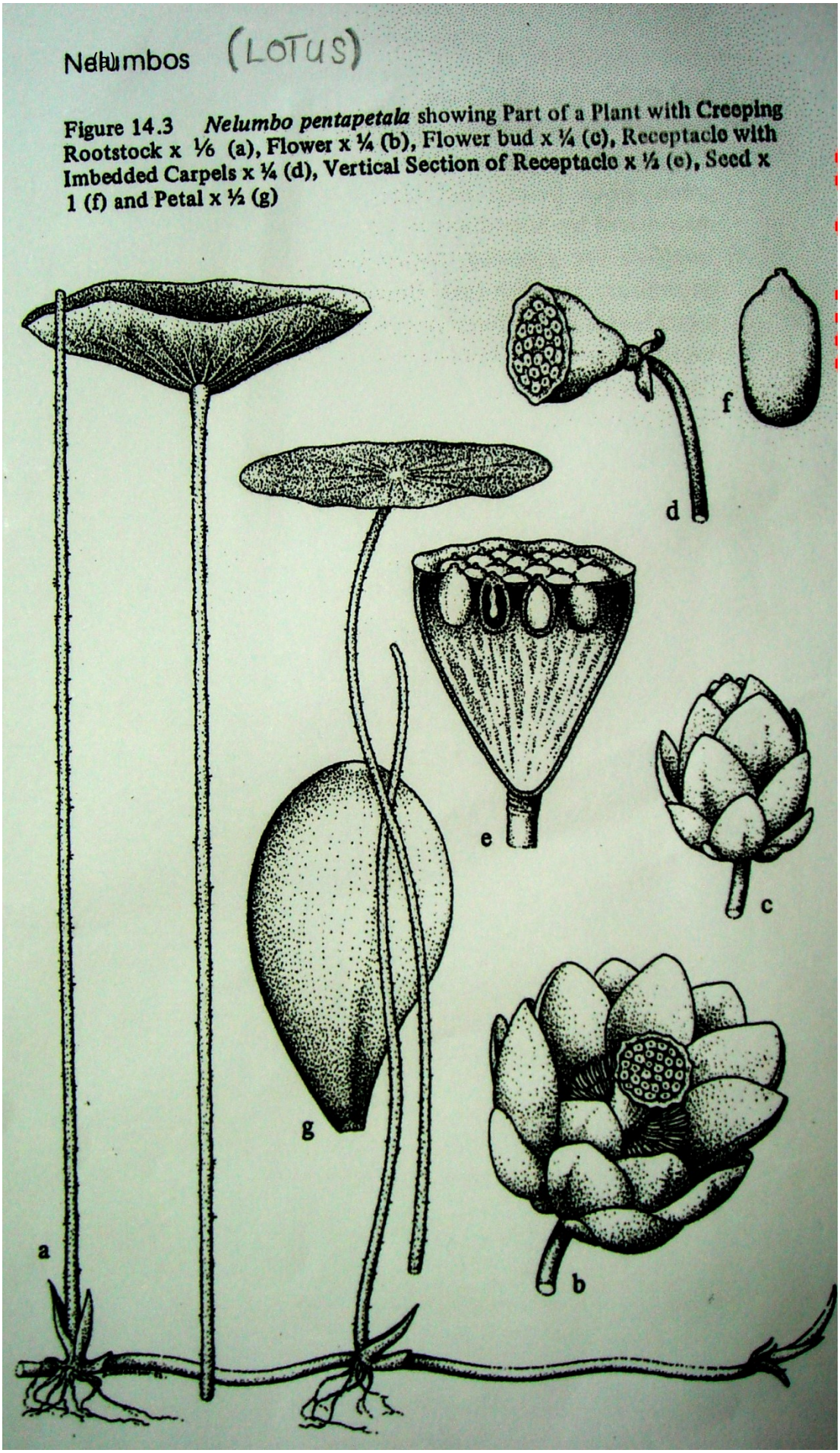
Table 1.1 : The physiological differences between Nelumbonaceae (lotuslily) and Nymphaeae (waterlily).

Parts	Nelumbonaceae (lotuslily)	Nymphaeae (waterlily)
Leaf	fully circular without notch 2 type of leaf;floating and emerging leaf no intermediate underwater leaves	float on the water surface a deep notch from the edge floating leaf only have intermediate underwater leaves
Stem	rough with small distinct prickles	Smooth
Medicinal properties	have psychoactive and anesthetic properties all parts are edible	have psychoactive and anesthetic properties not edible

Green seeds of lotus or known as crunchy fruits can be eaten raw with white cotyledons inside the green seedcoat. Between the cotyledons is the embryo which consist of two prominent inrolled leaves with attendant stem. When the seeds sprouts, the stems elongate and the leaves straighten up and unroll and push the inrolled leaves up to the water surface. Finally, they become the roundwater-repellent floating leaves emerged from the water level. This special character differentiate between lotuslily family (Nelumboceae) and waterlily family (Nymphaeae) as in Figure 1.5 and 1.6.

Nelumbos (LOTUS)

Figure 14.3 ***Nelumbo pentapetala*** **showing Part of a Plant with Creeping Rootstock x ⅙ (a), Flower x ¼ (b), Flower bud x ¼ (c), Receptacle with Imbedded Carpels x ¼ (d), Vertical Section of Receptacle x ½ (e), Seed x 1 (f) and Petal x ½ (g)**



(b)

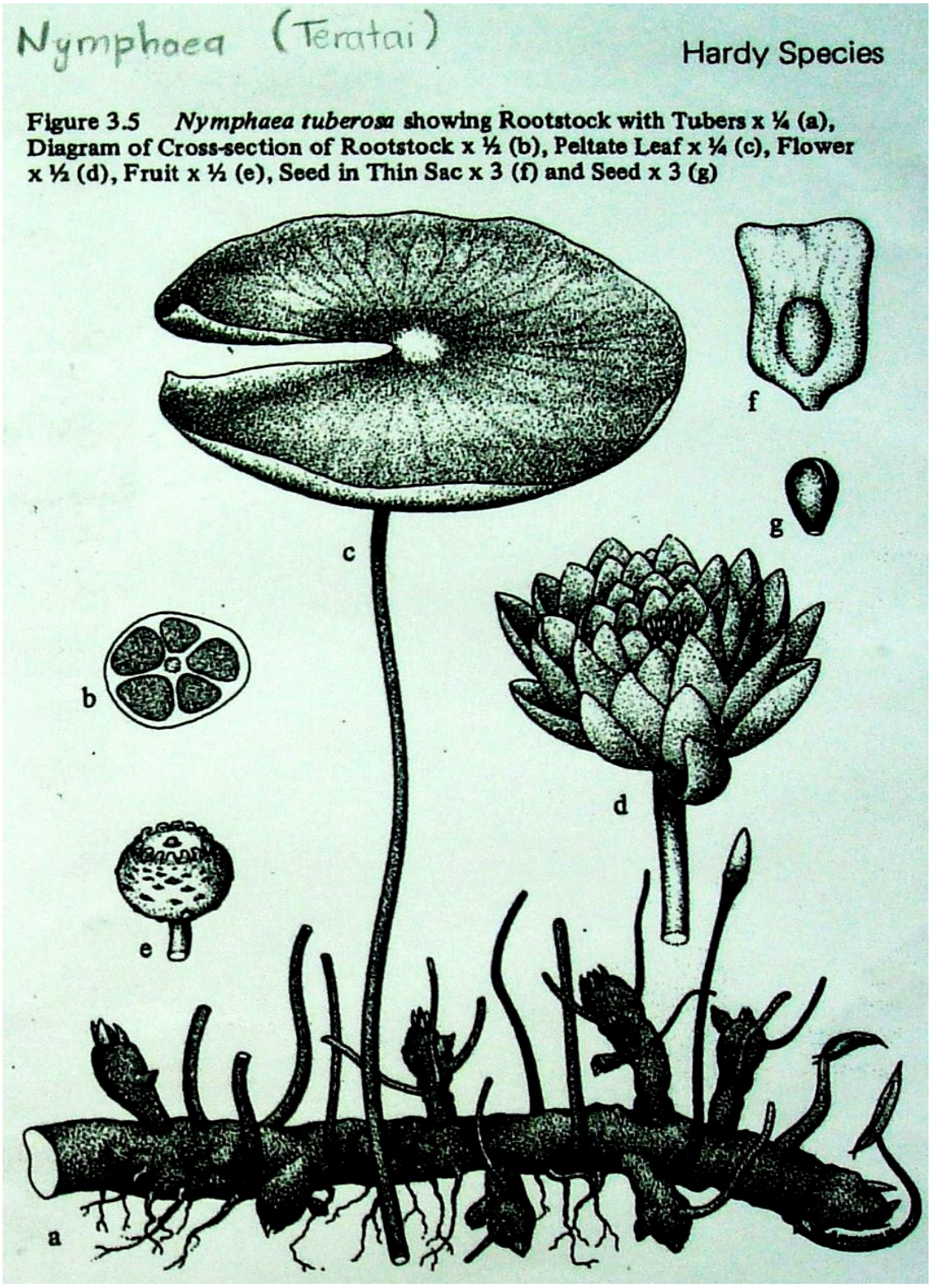


Figure 1.5 : Illustration by Swindells, P. (1983). Waterlilies. Timber Press, Portland, Oregon**.** (a) Lotuslily (b) waterlily.

Figure 1.6: (a) Pink Asian lotus flower Nelumbonaceae (lotuslily), (b) Pink Nymphaeae (waterlily).

1.1.3 Adaptations of Nelumbonaceae

The seed of the Lotus (*Nelumbo* sp.) is a very hard nut and is almost completely impermeable to water. It remains viable for many years; some evidence indicates over two hundred years at least. If the seed is placed into an ideal habitat for growth, it may still remain dormant for many decades before sprouting. It seems almost counterintuitive that being resistant to sprouting has a survival advantage.

Nelumbo seeds are hard nuts which are either round or oval with size of 0.5-2.0 cm (Figure 1.7). One end of the seed has a sharp point which is the remains of the floral stigma. The opposite end is a tiny dimple, a remnant of where the seed was attached to the mother plant. The seed color can vary from gray to dark brown or black. The shell is very hard and consists of two layers which are tightly bonded together. Inside the shell are two thin brown colored seed coats which enclose the twin cream colored cotyledons. This feature is what places the *Nelumbo* genus into the dicotyledon subclass of flowering plants (angiosperms). In the middle of the cotyledons is the *Nelumbo* embryo which consists of two prominent inrolled leaves with attendant stem. The leaves are doubled over against the stems because of the tight space.

The probable reasons for survival tactic in the genus *Nelumbo* are the two fold layers that tightly bonded together. This resistance to germination is caused by the seedcoat which is almost impermeable to water penetration. The secret for speeding up the germination process is to remove this protective cover without harming the internal seed. Many methods of doing this have been described in the literature including using

chemical solution such as soaking in concentrated sulfuric acid for 5 hours. Physical method such as rubbed along a rough surface to wear away part of the seed coat using medium grit sand paper or a concrete surface. The optimum grit size for the sandpaper is 80, although a finer grit (higher number) also helpful.

Figure 1.7 : Structure of *Nelumbo nucifera* Gaertn. matured seed (black seed) as the survival tactic for viability up to 200 years. Image by http://www.victoria-adventure.org/

Under suitable conditions for germination, the swelling of the seed to almost double in volume (Figure 1.8). This is due to the intake of water which hydrates the dried cotyledons and dormant embryo within the seed coat. In some cases, the seed coat will exhibit irregular bulges. The seed coat becomes soft and has the texture of leather. This process usually occurs within a day or two, but for some seeds it may take up to a week. The start of the sprouting is determined by the gradual splitting of the seed coat starting at the dimple end.

In a few cases, the seed may float to the surface of the water. These seeds will sprout just as readily as the ones that remain under water. A slight change in the color of the water in the container, either to a light tan or a cloudy white, the intensity being related to the amount of water in the container. The tan color comes from the tannin in the seed, the cloudiness from bacteria feeding on the other exudation from the seed interior.

Figure 1.8 : The swelling of the seed to almost double in volume within 2 months in water (a) 0.5 cm seeds (b) 1.0 cm swollen seeds.

After the swelling is completed, there will be a period of no activity situation (dormant). The sprouting periods can happen anywhere from one day to several weeks. When a seed does sprout, the seed coat splits longitudinally, starting from the dimple end. The two cotyledons then separate as if on a hinge at the pointed end of the seed. The folded-over stem of the green embryo can then be seen between them. The folded stem grows out of the seed shell and pulls the in rolled first leaf after it. At this stage of growth the seedling looks like a sharply bent fish hook with the inrolled leaves imitating the barb. The fish hook bend in the leaf stem slowly straightens out while the stem continues to grow until it is at least eight to fifteen inches long.

Consequently, because the stem is fairly stiff, the leaf may be pushed out of the water if the depth is less than eight inches. With a moderate water depth, the leaf stem will start to bend over and push the leaf horizontally just beneath the surface. For deeper water the stem will continue to grow vertically until the leaf reaches the surface. At that point, the inrolled leaf begins to grow and expand until it floats flat on the water. The leaf diameter will be between one and two inches. The period of time to reach this stage is about ten days after sprouting.

The seedling can be planted or potted after the formation of primary roots. Planting the seedling before or at the beginning of root growth prevents subsequent root damage. The seed contains enough food to sustain itself without extra nutrition until after the first four floating leaves have formed, about 30 days after the seed has sprouted. The preferred potting soil for *Nelumbo* seedlings is heavy loam which is more solid and compact to hold the roots.

In general, the *Nelumbo lutea* seed takes twice as long to sprout as the *Nelumbo nucifera* seed under similar conditions.

1.2 INTRODUCTION TO TISSUE CULTURE OF AQUATIC PLANTS

The history of plant tissue culture was started in 1838 when Schleiden and Schwan established the 'Theory of Cells'. According to this theory, every single cell is autonomic and thus, it is capable to develop into complete individual (totipotency). In 1901, the first attempt of plant tissue culture was done by Haberlandt, but failed. In 1934, White was successfully cultured *Lycopersicon esculentum* from root and meristem tissues.

During the past few years, there has been increasing interest in the use of aquatic vascular plants for the removal of pollutants from domestic and industrial sewage effluents (McDonald and Wolverton, 1976). Aquatic macrophytes can take up excessive nutrients and also play a crucial role in creating a favourable environment for a variety of chemical, biological and physical processes that contribute to the nutrient removal and degradation of organic compounds (Gumbricht, 1993; Chong *et al*, 2004). Of the several plants studied, lotus (*N. nucifera*) and water hyacinths (*Eichhornia crassipes*) are among the most commonly cited and appear to have the greatest potential for use in water pollution control and are known to accumulate nutrients (Hailer and Sutton, 1973; Ornes and Sutton, 1975; Cornwell et al., 1977).

In order to produce large numbers of these potential aquatic plants for this type of uses, one has to rely on vegetative propagation or sexual reproduction. Unfortunately, most aquatic plant species do not produce seeds and plants produced through asexual propagation are time consuming, labor intensive, expensive and not adequate to meet

the demands of industry (Thullen and Eberts, 1995). Furthermore, the harvesting of aquatic plants from their natural habitat will become a threat to the species richness (Lauzer, 2004).

Pierik (1987) reported that plants can propagate through sexual (generatively) or asexual (vegetatively) . The development of plant tissue culture is highly related to the chemical constituents which are the growth regulators (Skoog and Miller, 1957). This is important to determine the balance medium that is suitable for the growth of tissues from a particular plant type (de Klerk et al., 1999).

In vitro propagation or plant tissue culture is an alternative method in producing new plants (Chu and Kurtz, 1990). Through this cloning technique, plants with genetically identical to mother plant (true-to-type) can be produced by vegetative asexual propagation from small pieces of tissues (Bhojwani and Radzlan, 1983).

Therefore, tissue culture is a suitable method to mass propagate aquatic plant species and offers several advantages for industry. Among the advantages is good quality of planting materials with disease and virus free at a competitive price while conserving aquatic plants in their natural habitat. Large scale plant production also can be programmed and preservation of plant species *in vitro* is also possible. *In vitro* propagation is the most efficient and cost effective method of propagating large number of planting materials.

The plants produced by *in vitro* propagation are genetically uniform, vigorous and free from associations with other microorganisms and useful for the culture of aquatic plants where contaminating organisms can dominate other types of production systems (Alistock and Shafer, 2006). Many tissue cultured water plants show a more bushy growth with more adventitious shoots, qualities that many will appreciate (Christensen, 1996).

Plant tissue culture has become one of the most important prerequisites for successful genetic transformation, besides providing efficient *in vitro* micropropagation methods for many economically important plant species (Gamborg et al., 1968).

Tissue culture has been successfully employed for micro-propagation of a wide range of aquatic plants as stated in Table 1.2 but its application in lotus rarely reported possibly because its recalcitrance to regeneration *in vitro* (Zhao, 1999).

Table 1.2: Wide range of aquatic plant species that had been regenerated from tissue culture.

Species	Explants	Media	Source
Nelumbo lutea	Excised embryos from immature flowers	Half strength MS + 100mg inositol, 0.4mg thiamine, 100mg/l GA3	Kane et al. (1990)
Cryptocoryne lucen	Aerial plants	MS + 0.45mg BAP, 0.1mg NAA	Kane et al. (1990)
Myriophyllum aquaticum	Nodal and internode segments	Liquid half MS + 8mg/l 2iP	Kane et al. (1991)
Anubias barteri **var. *undulata***	Lateral shoots	MS + 0.3mg/l BA, 0.01 thiadiazuron and 0.1 NAA	Huang et al. (1994)
Trapa natans	Shoot tips and node	Nitsch's basal liquid medium (NBL) + 10^{-6}M BAP	Agrawal and Mohan Ram (1995)
Scirpus robustus	Seedling mesocotyl	MS + 1mg/l 2,4-D	Wang et al. (2004)

Recent advances in molecular biology, enzymology, physiology and fermentation technology of plant cell cultures suggest that these systems will become a viable source of important natural products.

1.2.1 Pigments and Pharmaceutical Values of *Nelumbo nucifera* Gaertn.

Natural pigments of plants are labile. The pigments can be easily altered, and even destroyed. On the basis of their chemical structures, pigments can be classed into four families, i.e. tetrapyrroles (e.g. chlorophyll), carotenoids (e.g. beta-carotene),

polyphenolic compounds (e.g. anthocyanins), and alkaloids (e.g. betalains) (Schoefs, 2004).

Plant carotenoids are red, orange and yellow lipid soluble pigments (Sun et al., 1996; Van den Berg et al., 2000) and these pigments are of important agronomic value in many crops and ornamental plants (Cunningham and Gantt, 1998). Up to now, more than 750 carotenoids have been isolated and identified from natural sources (Azevedo-Meleiro and Rodriguez-Amaya, 2005), but source material for their extraction is limited.

Plants produce biochemicals that are of importance in the healthcare, food, flavour and cosmetics industries. Whilst plant cell culture system or technology represent a potential renewable source of valuable medicinals, flavours, essences and colourants that cannot be produced by microbial cells or chemical syntheses. Currently, these and many other natural products are produced solely from massive quantities of whole plant parts.

Lotus is an important aquatic economic plant, not only as a dainty and ornamental flower but also as a source of herbal medicine with strong bioactive ingredients such as alkaloids, flavonoids, antioxidants, antisteroids, antipyretic, anticancerous, antiviral and anti obesity properties (Mukherjee et al., 1997; Sinha et al., 2000; Qian, 2002; Sridhar and Bhat, 2007).

According to different purposes or morphological differences, the Asian lotus (*N. nucifera* ssp. *nucifera*) is usually classified into three types: rhizome lotus, seed lotus and flower lotus. The rhizome and seeds of lotus are used as a vegetable, and the dried receptacle is used medicinally as a source of quercetin, a compound exhibiting anti-inflammatory and anti-oxidant activity.

The medicinal properties of lotus were recognized earlier than its cooking, and firstly recorded in the book ‘‘Er ya’’ (400 B.C.). It is estimated that 100 g fresh rhizome contains water 62.28–83.17 g, protein 1–3.86 g, starch 15–26.25 g, fat 0.1 g. Dry materials per 100 g includecoarse fibre 0.5 g, Ca 19mg, P 51 mg, Fe 0.5 mg,K 49.7 mg, Na 49.7 mg, Mg 16.4 mg, vitamin A0.02 mg,vitamin B1 0.11 mg, vitamin B2 0.14 mg and vitamin C 15–79.39 mg. Whilst, in dry seed per100 g contains starch 57.8–66.8 g, protein 16.6–19.0 g, total sugar 8.5–19.1g, and other amino acid, ions, vitamins (Li, 2007).

Lotus is identified as food and also as traditional medicine by the Chinese goverment (Chinese Ministry of Health, 2002). In Chinese Pharmacopoeia Committee (2005) reported that lotus seed (hold back diarrhoea), fruit (efficacy of hemostasia), stamen (prohibit pathological spermatorrhoea) and leaf (dispel thirsty) are listed as traditional medicine.

As home remedy (Figure 1.9), green lotus leaves are useful to treat summer heat syndrome in Japan and China and used to treat obesity in China (Ong, 1996). Pink petals of lotus are floated in soups or used as a garnish, while the yellow stamens are used in flavoring the tea. The roasted black seeds are good as coffee substitute and possess saponins, phenolics and carbohydrates in appreciable quantities. Pharmaceutical

value (Table 1.3) of medicinal parts of *Nelumbo nucifera* Gaertn. were described by Chinese Pharmacopoeia Committee (2005).

Table 1.3 : Valuable madicinal parts of *Nelumbo nucifera* Gaertn. (Pink Asian lotus).

PARTS	MEDICINAL ADVANTAGES	
	TRADITIONAL (By Ayurvedic medicinal)	PHARMACEUTICAL (By Chinese Pharmacopoeia Committee, 2005)
Pink Petal	Soup garnish	-
Yellow Stamen	Tea flavor	prohibit pathological spermatorrhoea
Green Fruit	Vegetable, nut	Efficacy of hemostasia
Black Seed	Coffee substitute	hold back diarrhoea
Creamy Rhizome	vegetable	-
Green Leaf	Treat heat and obesity	dispel thirsty

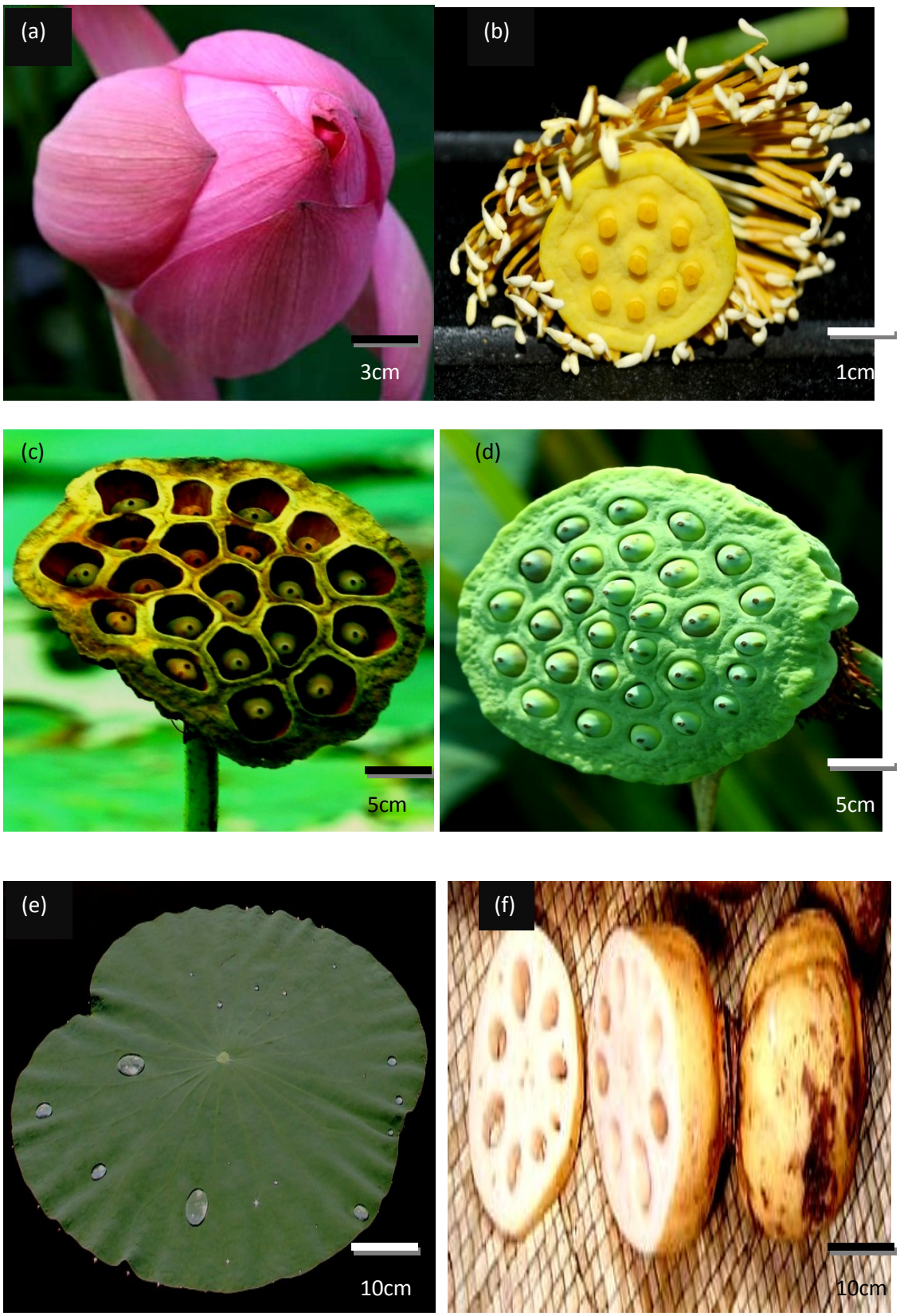


Figure 1.9: The multipurpose of lotus parts as nutritious and medicinal vegetable. (a) flower (b) stamen (c) seed (d) fruit (e) leaf (f) nodal rhizome.

There are more than 100 kinds resulting in a series of processed products from the conventional dry lotus seed, including preserved fresh lotus root, deepfreeze lotus root, juice of lotus root, starch of lotus root or seed, pickled lotus root, poached lotus root and tea of lotus leaf. Before the rhizome's swelling in May and June, the underground creeping stem of lotus can be harvested to eat as a flavorful food. Green lotus seed is a delicious food for leisure. The swollen stems are used for plant reproduction.

Nelumbo nucifera was reported to posses anti-diarrhoealpsychopharmacological, diuretic, antipyretic, antimicrobial, hypoglycemic activity and antioxidant activity (Hu, 2003), including all parts of the plant (Table 1.4). Traditional knowledge reveals the lotus edibility (nutritional values) and medicinal properties (pharmaceutical values). Lotus seeds are rich in proteins (10.6-14.8%) and essential minerals.

Lotus seeds are in high demand in Ayurvedic medicinal preparations and widely used in folk medicines to treat tissue inflammation, cancer and diuretics skin diseases and as poison antidote (Liu et al,. 2002). Seeds and roots of lotus are regarded as popular health food and the alkaloid (liensinine) extracted from them is effective to treat arrhythmia (Ling et al., 2006).

Table 1.4 : Valuable parts of *Nelumbo nucifera* Gaertn. for medicinal advantages.

Parts	Chemical compound	Uses	folkloric use (medicinal)
Roots		astringent	1.piles. 2. ringworm and other skin ailments (Paste of root starch)
rhizomes	Alkaloid, liensinine (Ling et al., 2005) ,Asparagin	astringent	rejuvenating tonic
Flowers/ petal	anthocyanins	astringent	1. diarrhea, cholera, liver complaints, and fevers. (Flowers, filaments and juice of flower-stalks) 2. syrup: coughs, beeding piles, menorrhagia and dysentery. 3. stop internal bleeding caused by gastric ulcers; menorrhagia or parturient hemorrhage. (Receptacle/.flower stalk) 4. premature ejaculation (Decoction of flowers)
Young leaves	Nelumbine Leaf;flavonoid,alkaloid.effective drugs		antifebrile and antihemorrhagic
leaves	chlorophyl	poultices	1.piles. 2. diarrhea 3. deterrent for skin maladies 4. high fevers, mucous membranes and skin irritation, and over the forehead for headaches. (Pounded leaves)-japan 5. treat obesity (china)
seeds	1.Nelumbine 2.Alkaloid, liensinine (Ling et al., 2005) 3.saponins, phenolics, carbohydrates (Anon, 1992) 4.seedembryo:benzoisoquinoline alkaloids-antidiabetic activity(Yi et al.,2007) 4.Flavonoid, alkaloid (Hu and Skibsted, 2002)	Poultices, coffee substitute (roasted)	leprosy and skin diseases; for spermatorrhea and erotic dreams. demulcent and nutritive
stamens	carotenoids	Perfumed tea	bleeding piles and parturition
Cotyledon	Nelumbine		antidiabetic activity

According to this special medicinal properties, in 2010, Chemical constituents and characteristics of lotus were classified and more studies have been done. *Nelumbo nucifera* was reported to possess anti-diarrhoeal, psychopharmacological, diuretic, antipyretic, antimicrobial, hypoglycemic activity and antioxidant activity.

There are several kinds of alkaloids available for treatment of cardiovascular disease in products of lotus root, cordon euryale, and arrowhead (Yuan et al., 2004; Yang, 2006; Meng et al., 2007). Products of aquatic vegetables generally rich in dietary fiber has been associated with lower levels of cardiovascular disease and cancer (Qian, 2002).

1.3 RESEARCH OBJECTIVES

Nelumbo nucifera Gaertn. (pink Asian lotus) was listed as one of the endangered aquatic species. Fortunately, Malaysian tropical climate with full sunlight throughout the year is suitable for vigorous propagation of lotus. Wetland or aquatic species contribute in decreasing global warming. The samples of lotus exclusively collected from the natural lake of Chini in Pahang, Eastcoast of Peninsular Malaysia. The special characteristic of the plant are magnificient in size of leaves and stem, while the number of seeds in the seed pod which contains more than 30-40 seeds compared to normal ponds (22-28 seeds), drains (14-18 seeds), containers or pots (8-14 seeds).

The objectives of this research are:

1. To establish an *in vitro* germination and micropropagation of *Nelumbo nucifera* Gaertn. through tissue culture system.
2 To develop an extraction method for pigment detection in *Nelumbo nucifera* Gaertn.
3. To determine an optimum condition for acclimatization of *in vit*ro lotus.

CHAPTER 2

SEED GERMINATION OF *Nelumbo nucifera* Gaertn.,

In Vivo and *In Vitro*

2.1 EXPERIMENTAL AIMS

The matured seeds of *Nelumbo* sp. (Figure 2.1) are large, hard, ovate, produced in a pithy receptacle. The black seeds are known as very thick and hard nut. It is completely impermeable to water penetration and seed longevity up to several hundred years, it remains viable for centuries (Hartman et al., 1990). Even though the seeds were placed into an ideal habitat for growth, it may still remain dormant for many decades before sprouting.

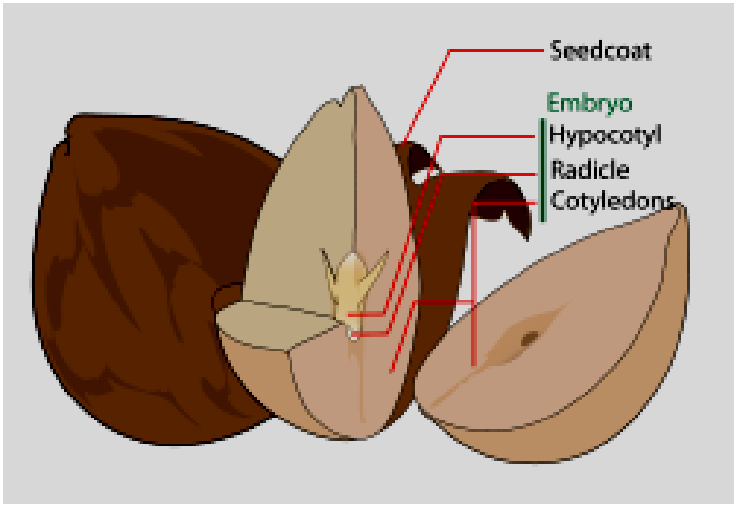


Figure 2.1 : General structure of a seed of *Nelumbo nucifera* Gaertn.

Lotus usually propagated vegetatively through rhizome division (Figure 2.2) or tuber production but normally with low propagation growth rate (Shou et al., 2008). It also can be multiplied through seeds but better germination will be obtained if the seeds are scarified by rubbing the outer hard seed coat gently on the sand paper at both ends and finally immersing in water to initiate germination. Scarified seeds will germinate after 3-4 days while normal seeds take 10-15 days to germinate. If the hard coating stays intact, the seed will remain viable for centuries and if placed in water it may take a few years for the seed to sprout (Hartman et al., 1990).

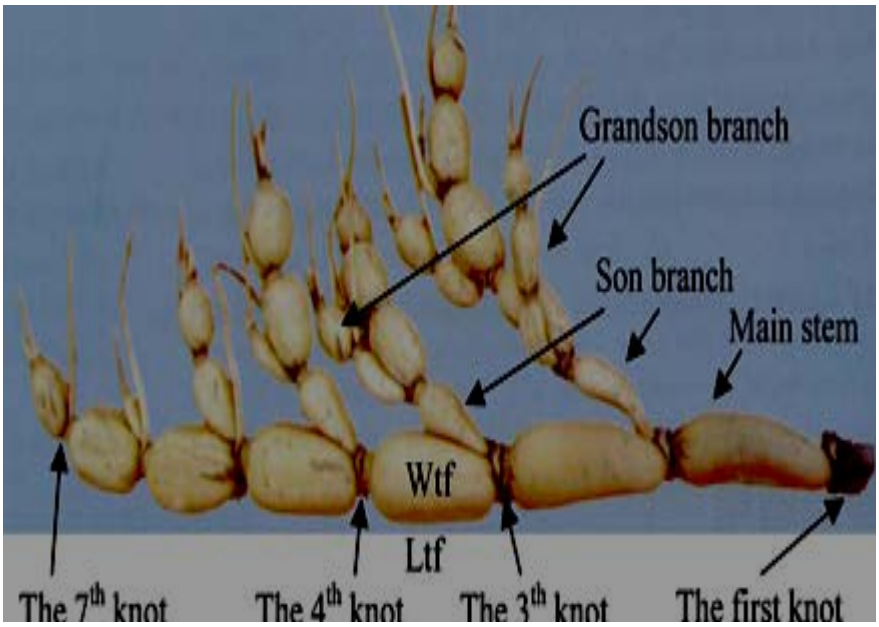


Figure 2.2 : General structure of rhizomes of *Nelumbo nucifera* Gaertn.

The rhizome and seeds of **lotus** are used as a vegetable and contain medicinal properties as a source of nelumbine and quercetin; the chemical compounds that exhibiting anti-inflammatory and anti-oxidant activity.

As the concern on germinating *in vivo* and *in vitro*, that affected by viability and storage of lotus seeds, the main objectives in this experiments were:

1. To identify the suitable technique for breaking dormancy in the lotus seed to obtain the highest germination rate *in vivo* and *in vitro* including mature, immature and young seeds.
2. To study the optimum condition for germinating lotus seeds and formation of roots on MS basal media.
3. To investigate the effect of low temperature storage through sodium alginate encapsulation (4±1°C) and frozen whole seeds (-20±1°C) method on germination rate of *Nelumbo nucifera* Gaertn..

2.2 MATERIALS AND METHODS

2.2.1 Source of Seeds

Wild seeds of *Nelumbo nucifera* Gaertn. were obtained from natural lake, Chini Lake in Pahang, Malaysia. Three types of seeds were collected from intact plants including matured seeds, immature seeds and young seeds.

2.2.2 Preparation for Breaking Seeds Dormancy

Lotus seeds are known as hardy nuts. Naturally, matured seeds (black) will sink in the pond and germinate when the conditions are suitable, which take several years. In this experiment, three types of seeds were used including matured seeds (black), immature seed or fruit (green) and young seeds (yellow).

As the pretreatment, seeds were rinsed with tap water and exposed to several techniques to break the seeds dormancy including using plain water, chemical solution with different soaking durations as below;

1. Soak in distilled water (control) for 24 hours
2. Soak in warm water (60°C) for 24 hours
3. Soak in distilled water for 2 months
4. Scarified with medium-grade sandpaper
5. Moist-prechilling in 4±1°C for 2 months
6. Soak in 0.1% (v/v) mercuric chloride for 2 minutes
7. Soak in 99% (v/v) chlorox for 2 minutes
8. Soak in 70% (v/v) ethanol for 2 minutes

2.2.3 Preparation of *In Vivo* and *In Vitro* Germination (Solid and liquid MS Media)

2.2.3.1 Sterillization of whole Seeds

Wild seeds of *Nelumbo nucifera* Gaertn. obtained from natural lake, Chini Lake in Pahang, Malaysia were initially washed with tap water and teepol. The seeds were then sterilized with 99% sodium hypochlorite added with two drops of tween twenty for 1 minute and rinsed with distilled water for three times. In the laminar flow, the seeds were dipped in 70% (v/v) ethanol for 1 minute and rinsed with sterile distilled water for three times and blotted on sterile tissue paper. These sterile and dried seeds were excised from the seed coat (coatless). These seeds were ready for culture on solid basal Murashige and Skoog (MS) media with thirty replicates in 60 ml sterile universal containers. All cultures were incubated in a growth room at 24±1°C, with a 16 hours photoperiod at 80-85 μmol m^{-2} s^{-1} under cool white fluorescent light (1000 lux).

2.2.3.2 Preparation of Solid MS Media

Distilled water (800ml) was added to ready-to-use 4.4g MS media powder from SIGMA, 30g sucrose and 8.0g technical agar (or 3.5g gelrite) in the 1000ml conical flask and constantly stirred by magnetic stirrer. The pH was adjusted to 5.8 by adding either 0.1 M sodium hydroxide (NaOH) or hydrochloric acid (HCl). Finally, the media was autoclaved at 104bkPa (15 Psi^2) at 121°C for 21 minutes.

2.2.4 *In Vitro* Germination on solid MS Media

The present study deals with *in vitro* germination of aquatic seeds (lotus) on solid MS media which useful for manipulation of the physiological and morphological characteristics. It is completely impermeable to water penetration and seed longevity up to several hundred years,The germination rates of *Nelumbo nucifera* were dependent on various factors including type of seeds (age of seeds) and pH. The seed with inner layer or coatless changed the white layer to brown and finally black because of the phenolic compound.

2.2.4.1 Identification of the Best Seeds Germination

Three types of seeds of different ages or maturity were studied. Oval black seeds were the matured seeds (15-20mm). Green seeds (fruit) with the same size had the smoother surface and easy to disinfectant. Yellow seeds from the flower pods were very small (2-5mm) and fragile with yellow embryo inside the cotyledon. The sterile dicotiledon seeds were excised into two. These coatless seeds without inner layer were cultured on MS basal media (without growth regulators/hormone) and incubated at 25±1°C with 16 hours light and 8 hours dark. Thirty replicates for each type of seeds were cultured.

2.2.4.2 Identification of the Optimum pH

Sterile and dried seeds were excised from the seed coats (coatless). These seeds were ready for culture on solid basal Murashige and Skoog (MS) media with thirty replicates each in five different pH (4.5, 5.5, 6.5, 7.5, 8.5) . All cultures were incubated in a growth room at 24±1°C, with a 16 hours photoperiod at 80-85 μmol m^{-2} s^{-1} under cool white fluorescent light (1000 lux).

2.2.5 Encapsulation Matrix

The development of encapsulated or synthetic seed technology brings up a new prospect in agriculture and nursery industry. Production of synthetic seeds is effective and acts as an important alternative method of propagation in commercially important and endangered plant species. Plants could be produced in large scale with high volumes. Consequently, genetic uniformity and stability of the plant could be maintained. Due to the sterility, it could be easily transported and has potential for long term storage without losing its viability. Up till now, regeneration of lotus from synthetic seeds has not been reported.

2.2.5.1 Preparation of 3% (w/v) Sodium alginate Solution ($NaC_6H_7O_6$)

Sodium alginate solution as an encapsulation matrix was prepared according to the heating method by Fabre and Dereuddre (1990) using 1%, 2%, 3%, 4% and 5% (w/v) of the concentrations. To prepare 1% (w/v) sodium alginate solution in 100ml MS basal medium without calcium chloride dehydrate ($CaCl_2.2H_2O$), 1g sodium alginate

powder were dissolved gradually. Sucrose (3.0g) was added and pH was set at 5.5. This solution was autoclaved for 21 minutes at 121°C and 104kpa.

2.2.5.2 Preparation of Calcium Chloride Dehydrate Solution ($CaCl_2.2H_2O$)

Calcium chloride dehydrate solution was used as a complexion agent. To prepare 75mM (w/v) calcium chloride dehydrate in 100ml distilled water, 1.47g $CaCl_2.2H_2O$ was dissolved gradually. This solution was autoclaved for 21 minutes at 121°C and 104kpa. Cold solution was used to soak in the beaded alginate in different time period.

2.2.5.3 Encapsulation Techniques and Bead Formation

Encapsulation matrix was introduced by Lynch (2002) and using micropipette as a tool for formation of beads with 5.0mm of pipette tip. However, in this research, sterile 5.0 ml syringe extended with 5mm pipette tip was used for beading. First, micro shoots sank in alginate solution. Then, 3ml of sodium alginate solution with one micro shoots will suck in the syringe before dropped in to calcium chloride dehydrate solution. Clear beaded automatically form and floating in the calcium chloride. Within 40 minutes, blur beaded will sink and ready to rinse 3 times with sterile distilled water. Beads were sieved using sterile nylon mesh and blotted with sterile tissue paper.

2.2.5.4 Low Temperature Storage

Sterile synthetic seeds (beads) were stored in the fridge at 4±1°C (low temperature) and covered with aluminium foil (dark condition). The survival rates for germination after for 15, 30, 45, 60, 75 and 90 days were recorded. Thirty replicates were used in each treatment.

Fresh seeds (green and yellow) were stored in freezer (-20°C±1°C) for 4 weeks. Juvenile shoots inside the seeds were excised and used in germination on MS basal media. Survival rates were recorded.

2.2.6 Data Analysis

All experiments were conducted using a completely randomized design. Data collected were statistical analyzed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at p=0.05.

2.3 RESULTS

2.3.1 Identification of the Highest Seeds Germination, *In Vivo* (in tap water) and *In Vitro (*solid MS Basal Media (1962))

Table 2.1 showed the responses and germination rates for three types of seeds including mature (black), immature (green) and young (yellow) due to eight various techniques in breaking the seeds dormancy in *vivo* and *in vitro*. The black and green seeds, both gave the highest response to technique of scarified with medium-sand paper. Germination from black seeds with rate of 67.01±0.28% *in vivo* and 78.35±0.61% *in vitro*. Whilst, germination from green seeds were higher with 75.12±0.16% *in vivo* and 100.00% *in vitro*. Moist-prechilling in 4±1°C for 2 months gave response lower than scarified seeds with 41.16±0.17% *in vivo* and 43.15±0.13% *in vitro* for black seeds (Figure 2.3), as well as 33.67±0.23% *in vivo* and 34.63±0.41% *in vitro* for green seeds, also 38.52±0.31% for yellow seed in *vitro*. While technique of soaking in 2 months distilled water were only effective to black seeds with swollen seeds at germination rate of 44.01±0.27% *in vivo* and 66.01±0.12% *in vitro*. Soaking for 2 minutes in 0.1% (w/v) mercuric chloride, 99% (v/v) chlorox and 70% (v/v) ethanol only gave response to green seeds with 26.31±0.23% *in vivo* and 33.33±0.17% *in vitro*, 23.33±0.21% *in vivo* and 25.06±0.13% *in vitro*, and 21.31±0.23% *in vivo* and 22.33±0.23% *in vitro*, respectively. Generally, both black and green seeds were responsive with highest germination rates in *in vitro* condition, while, yellow seeds were not responsive to all techniques *in vivo* and *in vitro*. No germination of whole seeds in soaking 24 hours in distilled water (control) were observed.

Table 2.1 : Responses of Different Seeds Maturity to Various of Break Seed Dormancy *in vivo* (in tap water) and *in vitro* (on solid Basal MS media with pH 5.5 and maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks).

Techniques	Seed Type	Observation	Germination Rate (%±SE)	
			In Vivo (in tap water)	*In Vitro* (on solid MS basal media)
Soak 24 hours in distilled water (control)	Black Green Yellow	No response No response No response	0 0 0	0 0 0
soak in warm water (60°C) for 24 hours	Black Green Yellow	Swollen seed Turn to black Turn to black	0 21.05±0.11e 0	0 23.33±0.21e 0
Soak 2 months in distilled water	Black Green Yellow	Swollen seed Turn to black Turn to black	44.01±0.27d 0 0	66.01±0.12b 0 0
Scarified with medium-grade sandpaper	Black Green Yellow	Swollen, cloudy water Turn to black Turn to black	**67.03±0.28c** 75.12±0.16b 0	**78.35±0.61b** 100.00±0.00a 0
Moist-prechilling in 4±1°C for 2 months	Black Green Yellow	Cracked seeds Cracked, black Turn to black	41.16±0.17c 33.67±0.23d 0	43.15±0.13d 34.63±0.41d 38.52±0.31d
Soak for 2 minutes in mercuric chloride	Black Green Yellow	No response Turn to brown Turn to black	0 26.31±0.23f 0	0 33.33±0.17d 0
Soak 2 min in 99% (v/v) chlorox	Black Green Yellow	No response Turn to brown Turn to brown	0 23.33±0.21e 0	0 25.06±0.13f 0
Soak 2 min in 70% (v/v) ethanol	Black Green Yellow	No response No response Turn to brown	0 21.31±0.23e 0	0 22.33±0.23e 0

Mean ± Standard error (SE), n=30. Mean with different letters in the same column differ significantly at p=0.05. The bold numbers represent the best result.

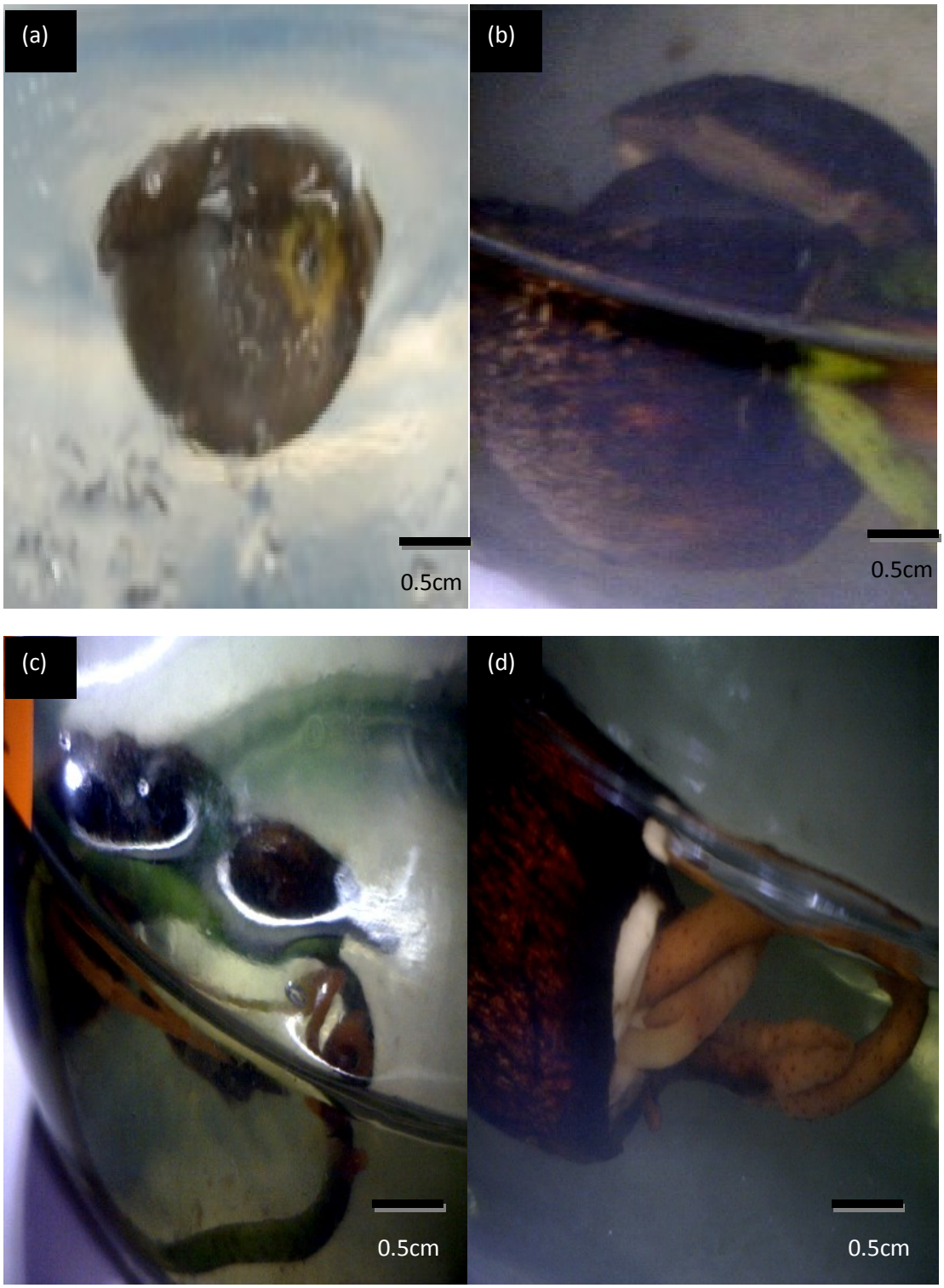


Figure 2.3 : The germination process of whole black seeds soaked in water for 2 months at 4±1°C (a) swollen moist-prechilling seed (b) cracked seed (c) shoot elongation (d) No root formation within 8-10 weeks.

2.3.2 Identification of the Best Excised Method for Seed Germination

Table 2.2 showed the responses of three types of lotus seeds on solid basal media with different methods of seed germination. Matured black seed pod (Figure 2.4 (a)), immature green pod (Figure 2.4 (b)) and young yellow pod (Figure 2.4 (c)) contains 8-40 seeds depends on the size of growth. The whole seeds without any scarified (Figure 2.4 (d)) as control (no response). The seed with inner layer or coatless (Figure 2.4 (g)) change the white layer to brown and finally black because of the phenolic compound to black, green and yellow seeds. The highest shoot length was for seeds without cotyledon (Figure 2.4 (f)) with 28.00±0.55mm for black seeds, 26.00±0.16mm for green seeds and 14.43±0.09mm for yellow seeds. Then followed by cultures whole seeds without inner layer (Figure 2.4 (e)) with 27.31±0.23mm for black seed and 25.12±0.11mm for green seed. The lowest shoot length was in seed with one cotyledon (Figure 2.4 (h)) with 15.33±0.23mm for black seed and 14.00±0.15mm. No shoot elongation for yellow seeds since the seeds turn to brown and died. No formation of roots for all seeds in this treatment. After 1 week on solid MS media, white cotyledon changed to green for both explants from green seed (Figure 2.4 (h)) and yellow (Figure 2.4 (i)),.

Table 2.2 : Responses of different germination methods in culturing black, green and yellow seeds on solid basal MS media with pH 5.5 and maintained at 25±1°C with 16 hours light and 8 hours dark, under 1000 lux intensity of light for 4 weeks.

Germination Methods	Type of Seeds	Observations	Shoot Length, mm(mean±SE)	No. Of root (mean ±SE)
Whole Seed (control)	Black	No response	0	0
	Green	No response	0	0
	Yellow	Yellow to brown	0	0
Whole seed with inner layer (coatless)	Black	White to brown, phenolic	0	0
	Green	White to brown, phenolic	0	0
	Yellow	White to brown, phenolic	0	0
Whole seed without inner layer	Black	White dried cotyledon	27.31±0.23a	0
	Green	White cotyledon to green	25.12±0.11b	0
	Yellow	Yellow cotyledon to green	14.33±0.47c	0
With one cotyledon	Black	Green shoots elongated	15.33±0.23c	0
	Green	Green Shoots elongated	14.00±0.15c	0
	Yellow	Yellow to green shoot	0	0
Without cotyledon	Black	Dark green shoots elongated.	**28.00±0.55a**	0
	Green		26.00±0.16a	0
	Yellow	White cotyledon changed to green (day 3-day 14) Formation of Primary roots	14.43±0.09c	0

Mean ± Standard error (SE), n=30. Mean with different letters in the same column differ significantly at p=0.05. The bold numbers represent the best result.

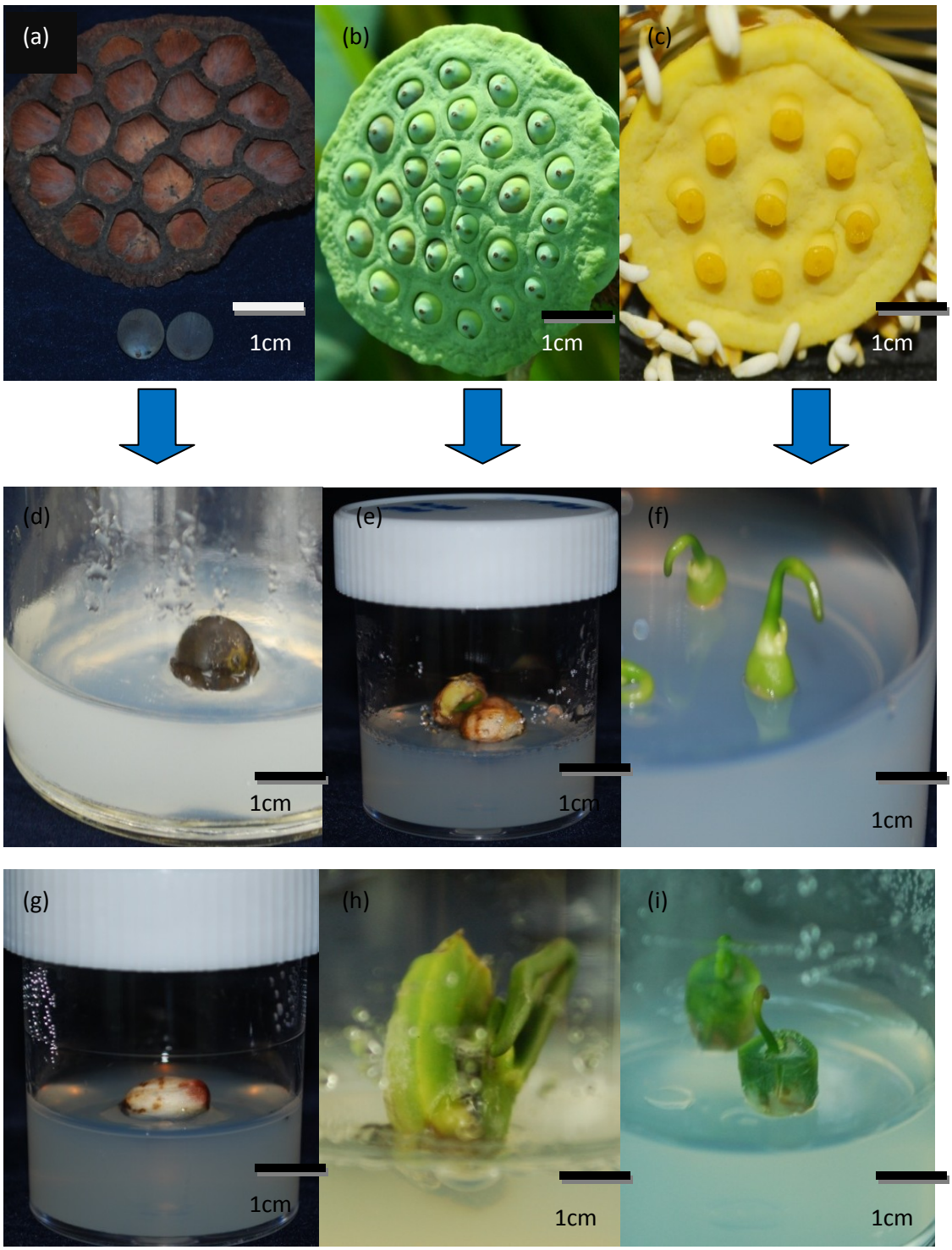


Figure 2.4 : Three types of seed sources germinated on solid MS basal Medium a) Black seed (mature) b) Green fruit (immature) (c) Yellow seed (young) (d) whole seed (e) Green seeds with one white cotyledon (f) yellow seeds without cotyledon turn to green within 6 days (g) whole seed with white inner layer (h) white cotyledon turn to green within 3 days (i) shoots elongation of yellow seed with green cotyledon.

2.3.3 Identification of the Optimum pH Seed Germination

Table 2.3 showed the effect of different pH in germination of seeds on solid MS basal media of three types of lotus seeds according to the age and maturity. Both black and green seeds responded to all of the media with pH 4.5, 5.5, 6.5, 7.5 and 8.5. The highest shoot length was in media with pH 5.5 for black seeds, green seeds and yellow seeds (Figure 2.5) and with 64.03±0.02 mm, 32.55±0.04 mm and 11.11±0.03 mm, respectively. These were followed by media with pH 4.5 with 50.01±0.23 mm for black seed and 26.88±0.51 mm for green seed. At pH 8.5, shoot length with the lowest with 22.31±0.02 mm and 11.33±0.31 mm were observed. However, young yellow seeds turned black and died.

Table 2.3: The Effect of Different pH on Seed Germination on solid MS basal media. Cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks.

pH	Shoot Length,mm (Mean±SE)		
	Black Seed	Green Seed	Yellow Seed
4.5	50.01±0.23b	26.88±0.51b	0
5.5	**64.03±0.02a**	**32.55±0.04a**	**11.11±0.03a**
6.5	38.15±0.32c	24.38±0.27b	0
7.5	25.64±0.17d	16.56±0.08c	0
8.5	22.31±0.02d	11.33±0.31d	0

Mean ± Standard error (SE), n=30. Mean with different letters in the same column differ significantly at p=0.05. The bold numbers represent the best result.

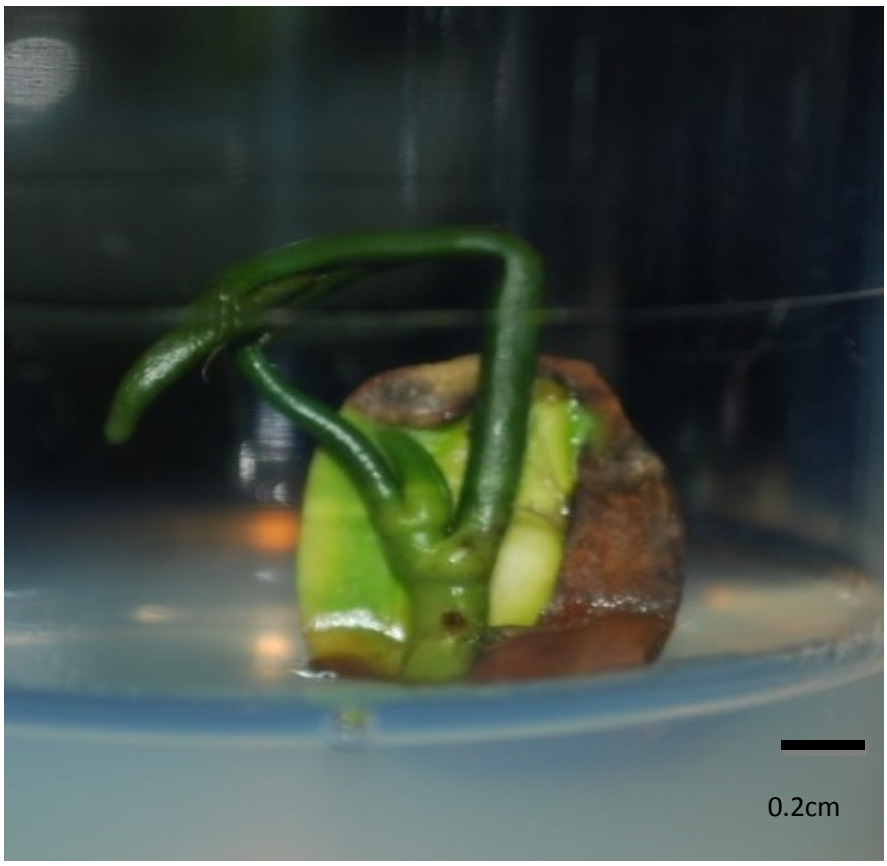


Figure 2.5: Shoots elongation from half cotyledon in media with pH 5.5.

2.3.4 Primary Root Induction Versus Time

Table 2.4 showed the response of primary root induction on solid MS basal media after 12 weeks in culture. All type of seeds responded to the media with 1mm root elongation per day. The maximum root length for both black and green seeds were 14mm on the 14th day with 3 green shoots and 6 white primary roots. While, maximum root length for yellow seeds was 17mm on the 18th day with 3 green shoots and 6 white primary roots. When the roots stopped elongation, dark green shoots became stunted. Figure 2.6 showed the root standard growth. From Figure 2.7, root formation started after 10-24 weeks in culture depending on the age of the seeds (a) 6-month-old multiple shoots (b) White primary roots (c) Roots elongation

Table 2.4 : The Response of root induction on solid MS basal media. Cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks.

Time (days)	Type of Seeds	Root Length (mm)	Observations
0 (control)	Black	0	Dark green plumule
	Green	0	Green plumule
	Yellow	0	Yellow plumule
2	Black	2	Thick, pink first primary root
	Green	2	Thick, pink first primary root
	Yellow	2	Thick, pink first primary root, green plumule
4	Black	4	White first primary root, pink second primarily root
	Green	4	White first primary root, pink second primary root
	Yellow	4	White first primary root, pink second primary root
6	Black	6	Roots elongation
	Green	6	Roots elongation
	Yellow	6	Roots elongation
8	Black	8	White first and second primary root, pink third primary root, green new shoot
	Green	8	White first and second primary root, pink third primary root, green new shoot
	Yellow	8	White first and second primary root, pink third primary root, green new shoot
10	Black	10	Roots and shoot elongation
	Green	10	Roots and shoot elongation
	Yellow	10	Roots and shoot elongation
12	Black	12	White first, second and third primarily root, shoot elongation
	Green	12	White first, second and third primarily root, shoot elongation
	Yellow	12	White first, second and third primary root, shoot elongation
14	Black	**14**	White first, second and third primary root, shoot elongation
	Green	**14**	White first, second and third primary root, shoot elongation
	Yellow	14	White first, second and third primary root, shoot elongation
16	Black	14	White first, second and third primary root, shoot elongation
	Green	14	White first, second and third primary root, shoot elongation
	Yellow	16	White first, second and third primary root, shoot elongation
18	Black	14	Stunted shoots
	Green	14	Stunted shoots
	Yellow	**17**	Shoots elongation
20	Black	14	Stunted shoots
	Green	14	Stunted shoots
	Yellow	17	Stunted shoots

Mean ± Standard error (SE), n=30. Mean with different letters in the same column differ significantly at p=0.05. The bold numbers represent the best result.

Root Length (mm)

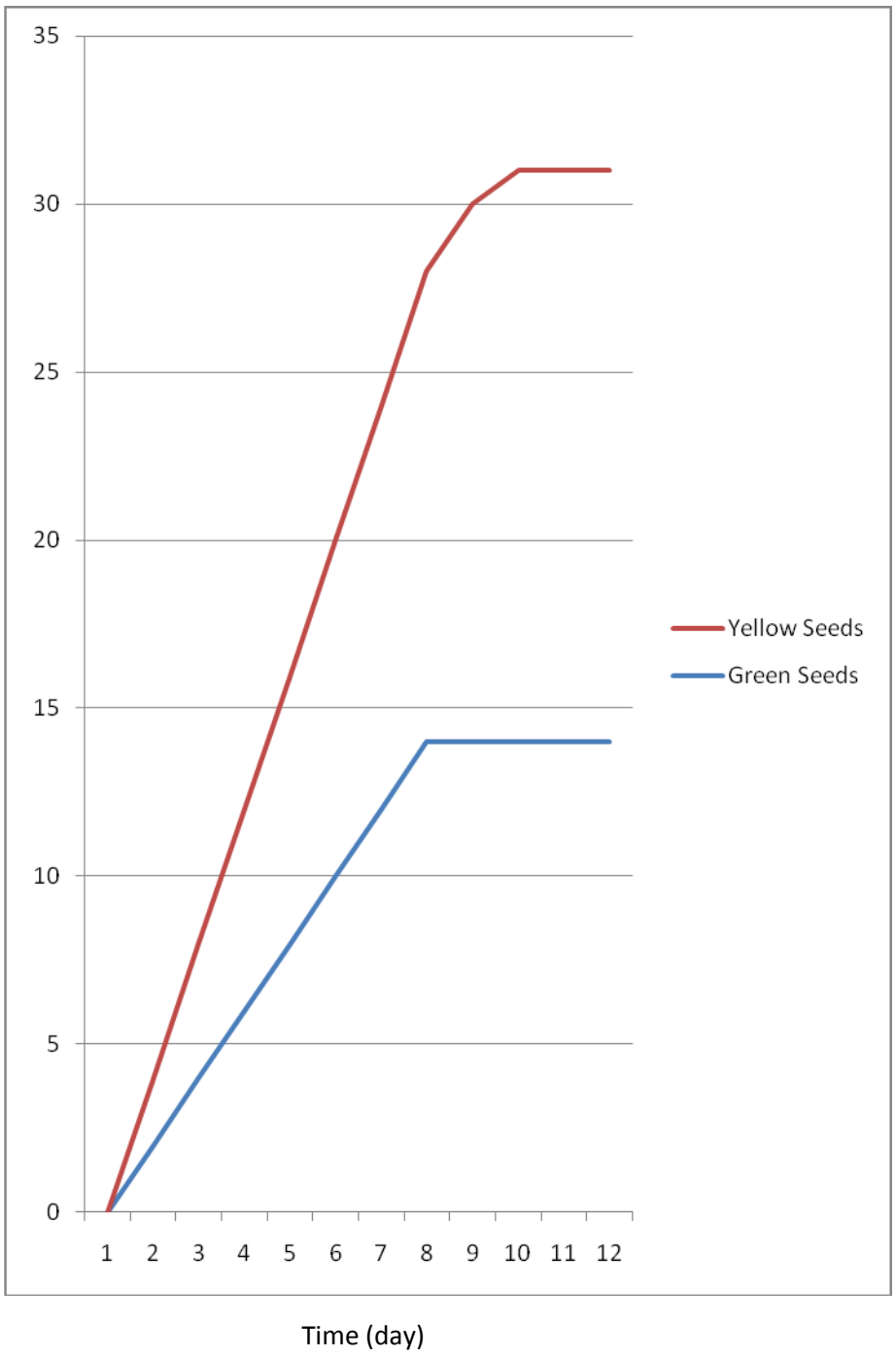


Figure 2.6: The standard root growth of *Nelumbo nucifera* Gaertn.

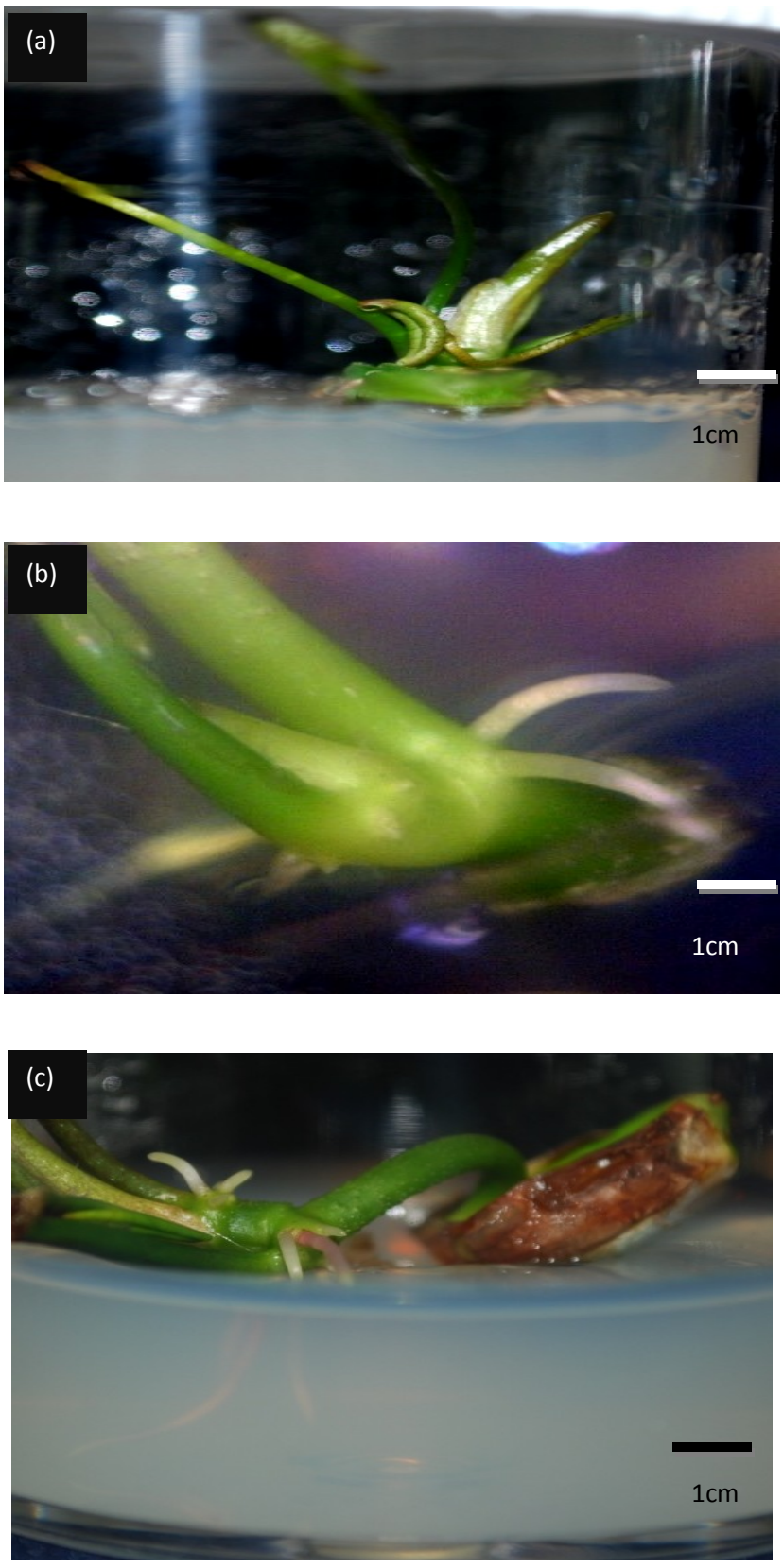


Figure 2.7: Root formation started after 10-24 weeks in culture depending on the age of the seeds (a) 6-months-old multiple shoots (b) white primary roots (c) roots elongation.

2.3.5 Identification of Suitable Sodium Alginate Encapsulated Matrix

Table 2.5 showed the effects of different concentration of sodium alginate ($NaC_6H_7O_6$) and soaking period of calcium chloride dehydrate ($CaCl_2.2H_2O$) on bead formation of lotus explants. With sterile condition, green plumules were excised from the seeds and encapsulated using sodium alginate solution for beads formation with ideal texture with uniform, isodiametric shape and size. The optimum concentration for the formation of encapsulation matrix was using 3.0% sodium alginate ($NaC_6H_7O_6$). Encapsulated explants were soaked in calcium 100 mM chloride dehydrate ($CaCl_2.2H_2O$) solution for 30 minutes. No suitable beads were formed with low concentration (1-2 %) of sodium alginate. Within 10 minutes (Figure 2.8 (a)), soaking in calcium chloride dehydrate, clear and beads formation with no definite shape. While, within 20 minutes (Figure 2.8 (b)) in calcium chloride dehydrate, beads were clear, solid and round at outside, however inside bead was still with liquid condition. The problem occurred when the bead cultured on solid MS basal media, the beads became shrunk and explants died. The optimum soaking period was 30 minutes (Figure 2.8 (c)) of calcium chloride dehydrate with high concentration of 3% sodium alginate which formed very hard beads but perfect round shape.

Table 2.5 : Effects of different concentrations of sodium alginate ($NaC_6H_7O_6$) and calcium chloride dehydrate ($CaCl_2.2H_2O$) on bead formation.

Soaking Period of $CaCl_2.2H_2O$ (minutes)	Concentration of $NaC_6H_7O_6$ (%)				
	1.0	2.0	**3.0**	4.0	5.0
10	-	+	++	++	++
20	-	+	++	++	++++
30	-	++	+++	++++	*
40	-	++	++++	++++	*

- No bead
+ Very fragile bead, no definite shape
++ Soft and oval bead
+++ Solid and round bead
++++ Hard and round bead
* Too hard and round bead

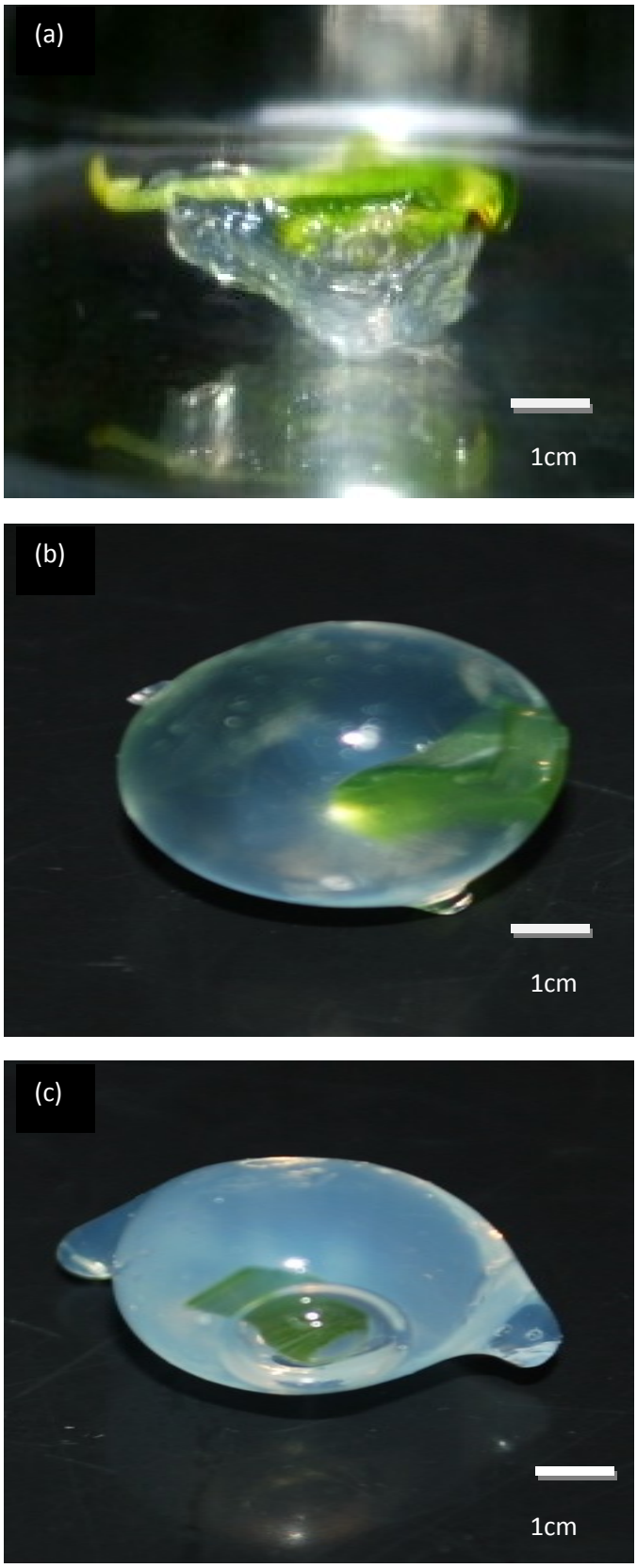


Figure 2.8 : Sodium alginate encapsulated shoots with different soaking period in calcium chloride dehydrate (a) 10 minutes; clear, soft and no definite shape (b) 20 minutes; clear, solid and round bead (c) 30 minutes; cloudy, solid and round bead.

2.3.6 Identification of Effect of Low Temperature Storage on Seeds Germination

Table 2.6 showed the effect of storage period on germination rate of *Nelumbo nucifera* Gaertn. with sodium alginate solution (Figure 2.9 (a)) at 4±1°C and frozen whole seeds at -20±1°C for 90 days. Initially, plumul inside the seeds were encapsulated in sodium alginate solution using sterile 5ml syringe (Figure 2.9 (b)). Then, beads were formed in calcium chloride. These beads were placed at 4±1°C with dark condition to avoid shoots elongation. The highest germination rate was on the first day of encapsulated with 100% germinated cultured on MS basal media (Figure 2.9 (c)). Beads were cracked (Figure 2.9 (d)) and shoots elongated (Figure 2.9 (e)). Germination rate was reduced to 98.73±0.51% after 15 days. After 90 days in storage (Figure 2.9 (f)), germination rate was only at 53.33±1.22%. Through frozen whole seeds method (-20±1°C), 100% germination rate until 60 days in storage. After 90 days in storage, the germination rate was still high with 93.32±0.63%.

Table 2.6 : Effect of storage period on germination rate of *Nelumbo nucifera* Gaertn at 4±1°C (sodium alginate encapsulated) and -20±1°C (frozen whole seeds) in 90 days.

	Encapsulated method (4±1°C)		Frozen method (-20±1°C)	
Period of storage (day)	No. Of germination (mean±SE)	Germination rate (%)	No. of germination (mean±SE)	Germination rate (%)
0	30.00±0.00	100.00±0.00	30.00±0.00	100.00±0.00
15	28.81±0.32	98.73±0.51	30.00±0.00	100.00±0.00
30	27.61±1.10	93.32±0.51	30.00±0.00	100.00±0.00
45	26.42±0.63	88.04±1.02	30.00±0.00	100.00±0.00
60	23.12±1.32	77.03±1.01	30.00±0.00	100.00±0.00
75	20.31±0.20	60.03±1.52	29.13±1.21	96.73±0.51
90	16.34±0.82	53.33±1.22	28.12±1.10	93.32±0.63

Mean ± Standard error (SE), n=30.

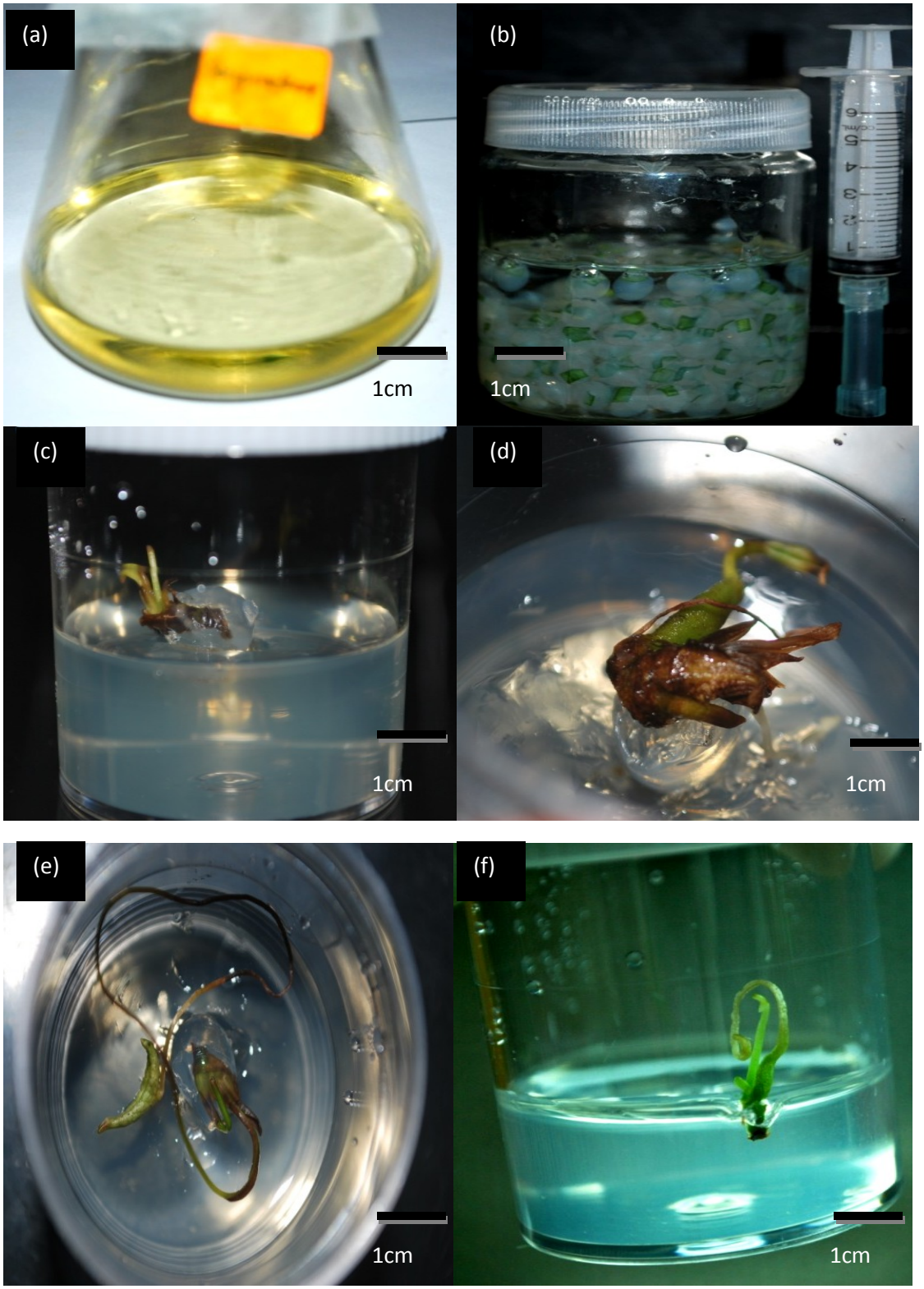


Figure 2.9 : Sodium alginate encapsulated shoots storage at 4±1°C (a) sodium alginate solution (b) encapsulated shoots (beading using syringe) soaked in calcium chloride for 30 minutes (c) bead placed on MS basal media (d) Cracked beads (e) Healthier shoot elongation after 15-45 days in storage (f) shoot elongated after 90 days in storage.

2.4 SUMMARY OF RESULTS

1. The black and green seeds both gave the highest response to technique of scarified with medium-sand paper and germinated in liquid and solid media. The black seeds with germination rate of 67.01±0.28% *in vivo* and 78.35±0.61% *in vitro* . While, green seed with 75.12±0.16% *in vivo* and 100.00% *in vitro*.

2. The highest shoot length from black and green seeds without cotyledon with 28.00±0.55 mm and 26.00±0.16 mm, respectively.

3. The highest shoot length was obtained in media with pH 5.5 for both black seed and green seeds and with 64.03±0.02mm and 32.55±0.04mm, respectively.

4. The maximum root length for both black and green seeds was 14mm on the 14^{th} day with 3 green shoots and 6 white primary roots. While, maximum root length for yellow seeds was 17mm on the 18^{th} day with 3 green shoots and 6 white primary roots.

5. The optimum concentration for the formation of encapsulation matrix was using 3.0% sodium alginate ($NaC_6H_7O_6$). Encapsulated explants were soaked in calcium 100 mM chloride dehydrate ($CaCl_2.2H_2O$) solution for 30 minutes.

6. Through sodium alginate encapsulated method (4±1°C), seeds germination reduced from 100% (day 1) to 53.3±1.2% (day 90)

7. Through frozen whole seeds method (-20±1°C), 100% germination rate was observed until 60 days in storage. After 90 days in storage, the germination rate was still high with 93.3±0.6 %.

CHAPTER 3

REGENERATION THROUGH TISSUE CULTURE SYSTEM OF *Nelumbo nucifera* Gaertn.

3.1 EXPERIMENTAL AIMS

Plant tissue culture refers to the growth and development of cells, tissues and organs which has been isolated from the mother plant and cultured *in vitro* on culture media under aseptic conditions (George, 1993). It provides efficient *in vitro* micropropagation methods for many economically important plant species (Gamborg, 2001) and the most important prerequisite for successful genetic transformation (Slater, 2008).

Tissue culture has been successfully employed for micropropagation of a wide range of aquatic plants, but its application in lotus rarely reported possibly because its recalcitrance to regeneration *in vitro* (Zhao, 1999). Tissue culture of lotus has been reported by researchers mainly from China, Japan and Thailand as a potentially alternative for lotus propagation.

Previous researches have proven that lotus can be propagated through tissue culture system. Liu (1948) investigated regeneration ability of excised lotus plumules. Francko (1986a) reported that germination of 98% in inoculated lotus seeds was obtained. The seedlings elongated and differentiated normally in sterile liquid culture. Shoots failed to directly generate from callus induced from immature embryos, green plumule leaves and young cotyledons, but could be directly induced from plumule leaves (Ke et al., 1987a). Shoots and plantlets were successfully obtained through stem tip culture (Yamamoto and Matsumoto, 1986, 1988; He and Liu, 1987). According to Arunyanart (1998), regeneration was successfully achieved from lotus flowers and apical buds were more efficient than axillary buds for shoot induction (Luo et al., 2004a). Callus was induced from buds, cotyledons and young leaf explants on Murashige and Skoog medium (1962), and somatic embryos were successfully induced from callus (Arunyanart and Chaitrayagun, 2005). An *in vitro* multiplication of lotus through shoot proliferation from underground rhizomes has been reported (Shou et al., 2008).

The main objectives of this research on *Nelumbo nucifera* Gaertn. were:

i) To identifiy the optimum media for shoot and root formation with different concentrations and combinations of BAP and NAA on 2 types of explants; green shoot (mature) and yellow shoots (juvenile).

ii) To explore the potential of rapid and mass propagation of *Nelumbo nucifera* Gaertn. from immature and mature explants.

iii) To study the effect of MS media strength, pH, light distance and double layer media (ratio liquid to solid) on lotus regeneration.

3.2 MATERIALS AND METHODS

3.2.1 Sterilization Protocol for Aseptic Seedlings

These seeds were initially washed with tap water and teepol. The seeds were then sterilized with 100% sodium hypochlorite and added with two drops of tween twenty for 1 minute and rinsed with distilled water for three times. In laminar flow, the seeds were dipped in 70% (v/v) ethanol for 1 minute and rinsed with sterile distilled water for three times and blotted on sterile tissue paper. These sterile and dried seeds were excised and removed from the seed coat (coatless). These seeds were ready for culture on solid basal Murashige and Skoog (MS) media with thirty replicates in 60ml sterile universal containers. All cultures were incubated in a growth room at 24±1°C, with a 16-h photoperiod at 80-85 μmol m^{-2} s^{-1} under cool white fluorescent light (1000 lux).

3.2.2 Preparation of *In Vitro* Explants

From chapter 2, 2-week-old green petioles from aseptic seedlings were cut into small pieces (3mm^2) and horizontally and abaxial surface down, cultured on solid MS media, pH 5.5 with 30 different concentrations of hormone combinations NAA and BAP. Thirty replicates for each treatment were used. Every 3-4 weeks the *in vitro* plants were subculture on the same media. Simultaneously, as comparison explants from leaf, stem and young flower pods from intact plants were cultured on the same media. All sterile containers were labeled with name of species, type of explants, date of culture and treatments of media.

3.2.3 Preparation of Solid MS Basal Media (1962) with Hormones

800ml distilled water was added with ready-to-use 4.4g MS media powder from SIGMA, 30g sucrose and 3.5g gelrite powder in the 1000ml conical flask and placed on hot plate until boil. Constantly stirred by magnetic stirrer. Hormones with combination BAP and NAA were added when the solution at 60°C. The pH was adjusted to 5.5 (according to the result from chapter 2) by adding either 0.1 M sodium hyroxide (NaOH) or hydrochloric acid (HCl). Finally, the media was autoclaved at 104bkPa (15 Psi^2) at 121°C for 21 minutes. Alternatively to instant MS powder, a preparation of MS Stock Solution (Apendix 1) containing macronutrients, micronutrients, iron, vitamin and myo-inositol.

3.2.4 Preparation of Hormones Stocks

NAA and BAP dissolved in NaOH or alchohol. Hormones that sensitive to the heat will not be autoclave. These hormones were dissolved with NaOH or alcohol and filtered with 0.22 µm sterile membrane filters (milipore filter) in laminar flow. Filtered hormones were added to the media while the media was at molten phase (50°C).

3.2.5 Sterile Culture Conditions

All apparatus including forceps, scalpel, tissue and others were wrapped in aluminium foil and autoclaved for 21 minutes at pressure of 104kPa (15Psi^2) and temperature of 121°C. Laminar flow chamber need to expose to UV light for 20 minutes before use. Then 70% (v/v) ethanol was sprayed to the surface and clean with sterile tissue towel. While, hot bead at 250°C was used to dip forceps and scalpel before being cooled with sterile distilled water. Then the cold apparatus were used to cut the explants tissue and directly transferred to media in the sterile tubes. All cultured explants were

incubated in the culture room at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux light intensity of light.

3.2.6 Subculture

Subculture was done every 21-28 days to provide new nutrient and fresh nutrient under the same condition.

3.2.7 *In Vitro* Cultures of *Nelumbo nucifera* Gaertn.

Tissue culture methods for the selection of variant types in ornamentals has been documented for many years especially for flower colour, plant morphology and also physiological characters. *In vitro* methods has known to shorten breeding cycles and therefore reduced the costs of the development of a new cultivar. A few factors that need to be considered in selection of explants in tissue culture are source of explants, (intact or aseptic plants), explants size, ontogeny, age of explants or the maturation of plant stock. Besides factors that are related to explant tissues, there are also other factors that play important role in the success of micropropagation including the addition of the growth regulators (plant hormone).

3.2.7.1 Induction of Shoots and Roots Formation on Solid Media with Combinations of BAP and NAA

Juvenile shoots (3 mm^2) from 2-week-old seeds (green and yellow) were cultured on solid MS media supplemented with 30 combinations of BAP and NAA at different concentration . Thirty replicates were used in every combination as below:

1. MS media without hormones (control)
2. MS + 0.5 mg/l BAP + 0.5 mg/l NAA
3. MS + 0.5 mg/l BAP + 1.0 mg/l NAA
4. MS + 0.5 mg/l BAP + 1.5 mg/l NAA
5. MS + 0.5 mg/l BAP + 2.0 mg/l NAA
6. MS + 0.5 mg/l BAP + 2.5 mg/l NAA
7. MS + 1.0 mg/l BAP + 0.5 mg/l NAA
8. MS + 1.0 mg/l BAP + 1.0 mg/l NAA
9. MS + 1.0 mg/l BAP + 1.5 mg/l NAA
10. MS + 1.0 mg/l BAP + 2.0 mg/l NAA
11. MS + 1.0 mg/l BAP + 2.5 mg/l NAA
12. MS + 1.5 mg/l BAP + 0.5 mg/l NAA
13. MS + 1.5 mg/l BAP + 1.0 mg/l NAA
14. MS + 1.5 mg/l BAP + 1.5 mg/l NAA
15. MS + 1.5 mg/l BAP + 2.0 mg/l NAA

16. MS + 1.5 mg/l BAP + 2.5 mg/l NAA

17. MS + 2.0 mg/l BAP + 0.5 mg/l NAA

18. MS + 2.0 mg/l BAP + 1.0 mg/l NAA

19. MS + 2.0 mg/l BAP + 1.5 mg/l NAA

20. MS + 2.0 mg/l BAP + 2.0 mg/l NAA

21. MS + 2.0 mg/l BAP + 2.5 mg/l NAA

22. MS + 2.5 mg/l BAP + 0.5 mg/l NAA

23. MS + 2.5 mg/l BAP + 1.0 mg/l NAA

24. MS + 2.5 mg/l BAP + 1.5 mg/l NAA

25. MS + 2.5 mg/l BAP + 2.0 mg/l NAA

26. MS + 2.5 mg/l BAP + 2.5 mg/l NAA

27. MS + 3.0 mg/l BAP + 0.5 mg/l NAA

28. MS + 3.0 mg/l BAP + 1.0 mg/l NAA

29. MS + 3.0 mg/l BAP + 1.5 mg/l NAA

30. MS + 3.0 mg/l BAP + 2.0 mg/l NAA

31. MS + 3.0 mg/l BAP + 2.5 mg/l NAA

All cultures were maintained at 25±1 °C with 16 hours light and 8 hours dark in the culture room. The cultures were observed and data were collected weekly.

3.2.7.2 Identification of the Optimum MS Strength

Juvenile shoots inside the cotyledon from green and yellow seeds were excised (2-5mm^2). These tissues were cultured on optimum media for regeneration with different strength including 0 g/l MS, 2.2g/l MS (½ strength), 4.4 g/l MS (full strength), 6.6 g/l MS (1 ½ strength) and 8.8 g/l MS (double strength). Cultures were incubated at 25±1°C with 16 hours light and 8 hours dark. Thirty replicates for each type of MS strength were used.

3.2.7.3 Determination of the Optimum pH

Juvenile shoots inside the cotyledon from green and yellow seeds were excised into 2-5 mm^2 in size. These tissues were cultured on optimum media for the regeneration with different pH (4.5, 5.5, 6.5, 7.5, 8.5). All cultures were incubated at 25±1°C with 16 hours light and 8 hours dark. Thirty replicates for each treatment were prepared.

3.2.7.4 Effects of Distance of the Light Source

Cultures on the optimum media which were identified as the best MS strength and pH were exposed to the light source at different distances (50.0 cm, 10.0 cm, 15.0 cm, 20.0 cm, 25.0 cm and 30.0 cm). Cultures were incubated at 25±1°C with 16 hours light and 8 hours dark. Thirty replicates for each distance were prepared.

3.2.7.5 Effects of Double-layered Liquid and Solid Media

Multiple shoots from the optimum media were subcultured every 21-28 days to supply fresh nutrients. Plantlets with shoots and roots were cultured on hormone solid media (bottom layer) added liquid MS basal (upper layer). Solid media were fixed to 1.0 cm height in the sterile tubes. Liquid media level from ratio 1:1, 1:2 and 1:3 to the solid level. Thirty replicates for each treatment were used.

3.2.8 Data Analysis

All experiments were conducted using a completely randomized design. Data collected were statistical analyzed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at $p=0.05$. Thirty replicates for each treatment were prepared.

3.3 RESULTS

3.3.1 Shoot and Root Formation on Solid Media with Combinations of BAP and NAA

Table 3.1 showed the responses of juvenile shoots from green and yellow seeds on 30 different combinations and concentrations of BAP and NAA of solid MS media. For green seed explants, shoots elongated (Figure 3.2 (a)) within 2 weeks. Rolled leaf (Figure 3.2 (b)) formed in week 12, open or unrolled leaf (Figure 3.2 (c)) after another 4 weeks. The highest number of shoots per explant for green seeds were on MS media supplemented with combinations of 1.5 mg/l BAP and 0.5 mg/l NAA with 10.33 ±0.23 shoots per explant (Figure 3.2 (d)). The lowest number of shoots per explant for green seeds were on MS media supplemented with combinations of 3.0 mg/l BAP and 2.5 mg/l NAA with 1.33 ±0.23 shoots per explant slightly lower than in MS without hormones, 1.43 ±0.10 shoots per explants. Only four treatments were successfull for root formation (Figure 3.1 (b)). The highest number of roots per explant for green seeds explants was on MS basal media with 4.33 ±0.53 shoots per explant. Followed by 1.5 mg/l BAP 0.5 mg/l NAA, 1.5 mg/l BAP 1.5 mg/l NAA and 1.0 mg/l BAP 2.5 mg/l NAA with 3.67 ±0.32 roots per explant, 0.67 ±0.09 roots per explant and 0.57 ±0.35 roots per explant, respectively. Some formation of abnormal (red and oval) shoots occurred in MS media supplemented with combinations of 1.0 mg/l BAP and 2.5 mg/l NAA, 2.5 mg/l BAP and 2.5 mg/l NAA, 1.5 mg/l BAP and 2.0 mg/l NAA and 1.5 mg/l BAP and 2.5 mg/l NAA (Figure 3.4).

Figure 3.1 (a) showed the responses and comparison on 30 different combinations and concentrations of BAP and NAA on juvenile shoots from green and

yellow seeds. The highest number of shoots per explant for yellow seeds were on MS media supplemented with combinations of 0.5 mg/l BAP and 1.5 mg/l NAA with 16.00±0.30 shoots per explants (Figure 3.3 (a)). Whilst, no root formation in all treatments for yellow seed explants (Figure 3.3 (d)) . Within the same period (1-24 weeks) of culture on solid MS media, regeneration from green seeds explants were exactly the same as mother plant with shoots and roots (complete plantlets), while regeneration from yellow seeds were different with smaller shoots elongated (Figure 3.3 (a)) pinkish rolled leaf (Figure 3.3 (b)), thin layered shoots (Figure 3.3 (c)) and without roots. No abnormal shoot formation from yellow seed explants.

Table 3.1 : The Responses on Different Combinations and Concentrations Of BAP and NAA on Juvenile Shoots from Green and Yellow Seeds, Cultured on Solid MS Media at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 24 weeks.

MS+Hormone		Explant	Observations	No. of shoots per explant (mean±SE)	No.of Roots per explant (mean±SE)
BAP (mg/l)	NAA (mg/l)				
0.0	0.0	Yellow **Green**	No response Shoots elongation, roots formation after 4 weeks Adventitious roots after 6 weeks	0 1.43 ±0.10abc	0 **4.33±0.53d**
0.5	0.5	Yellow Green	Yellow shoots to green after 1 week Shoots elongated	6.00±0.31ef 3.00 ± 0.26e	0 0
0.5	1.0	Yellow Green	Shoots yellow to green after 1 week Shoots elongated	8.00±0.00h 3.67±0.32fg	0 0
0.5	1.5	**Yellow** Green	Layered green shoots after 7 weeks Shoots elongated	**16.00±0.30m** 4.33±0.23gh	0 0
0.5	2.0	Yellow Green	Yellow shoots elongated Shoots elongated	2.67±0.18bc 2.67±0.23de	0 0
0.5	2.5	Yellow Green	Yellow shoots elongated Shoots elongated	2.67±0.67bc 2.10 ±0.32bcde	0
1.0	0.5	Yellow Green	Shoots yellow to green after 1 week	14.20±0.73l 6.00 ±0.15jk	0 0
1.0	1.0	Yellow Green	Shoots yellow to green after 1 week Shoots elongated	11.33±0.17k 4.00 ±0.40g	0 0
1.0	1.5	Yellow Green	Yellow shoots elongated Multiple shoots and creepers formation after 8 weeks	5.77±0.34e 6.33± 0.23k	0 0
1.0	2.0	Yellow Green	Shoots yellow to green after 1 week Multiple shoots and creepers formation after 8 weeks	10.67±0.18jk 6.33 ±0.17k	0 0
1.0	2.5	Yellow Green	Shoots yellow to green after 1 week Shoots elongated	6.00±0.30m 2.67 ±0.23de*	0 0.57 ±0.35a
1.5	0.5	Yellow **Green**	Yellow shoots elongated Multiple shoots and creepers formation after 4 weeks	3.67±0.32d **10.33 ±0.23m**	0 3.67 ±0.32c
1.5	1.0	Yellow Green	Shoots yellow to green after 1 week Multiple shoots and creepers formation after 6 weeks	9.33±0.18i 8.67 ±0.18l	0 0
1.5	1.5	Yellow Green	Shoots to green Multiple shoots	10.33±0.88j 5.33 ±0.38ij	0 0.67 ±0.09b

'Table 3.1, continued'

1.5	2.0	Yellow Green	Yellow shoots elongated Shoots elongated	2.00±0.00b 2.33 ±0.32cde*	 0
1.5	2.5	Yellow Green	No response Shoots elongated	0 2.33 ±0.32cde*	0 0
2.0	0.5	Yellow Green	Shoots yellow to green after 1 week Multiple shoots and creepers formation after 4 weeks	7.33±0.18gh 8.00 ±0.40l	0 0
2.0	1.0	Yellow Green	Shoots yellow to green after 1 week Multiple shoots and creepers formation after 4 weeks	8.00±0.00h 6.33 ±0.31k	0 0
2.0	1.5	Yellow Green	Yellow shoots elongated Multiple shoots in week 8	6.00±0.00ef 5.33 ±0.09ij	0 0
2.0	2.0	Yellow Green	Yellow shoots elongated Shoots elongated	5.33±0.35e 1.07 ±0.15a	0 0
2.0	2.5	Yellow Green	Yellow shoots elongated Multiple shoots and creepers formation after 8 weeks	3.33±0.35cd 5.67 ±0.17ijk	0 0
2.5	0.5	Yellow Green	Yellow shoots elongated Shoots elongated	6.07±0.41ef 3.67 ±0.18fg	0 0
2.5	1.0	Yellow Green	Yellow shoots elongated Shoots elongated	3.33±0.18cd 4.00 ±0.40g	0 0
2.5	1.5	Yellow Green	Yellow shoots elongated Shoots elongated	2.00±0.00b 5.00 ±0.46hi	0 0
2.5	2.0	Yellow Green	shoots elongated Multiple shoots	2.00±0.00b 6.33 ±0.35k	0 0
2.5	2.5	Yellow Green	shoots elongated Shoots elongated	2.00±0.00b 2.33 ±0.35cde*	0 0
3.0	0.5	Yellow Green	shoots elongated Multiple shoots	6.67±0.18fg 4.33 ±0.31gh	0 0
3.0	1.0	Yellow Green	shoots elongated Multiple shoots	2.67±0.18bc 4.33 ±0.35gh	0 0
3.0	1.5	Yellow Green	shoots elongated Shoots elongated	2.00±0.00b 2.00 ±0.26bcd	0 0
3.0	2.0	Yellow Green	shoots elongated Multiple shoots	2.00±0.00b 5.00 ±0.26hi	0 0
3.0	2.5	Yellow Green	No response Shoots elongated	0 1.33 ±0.23ab	0 0

*Formation of abnormal shoots

Mean ± Standard error (SE), n=30. Mean with different letters in the same column differ significantly at p=0.05. The bold numbers represent the best result.

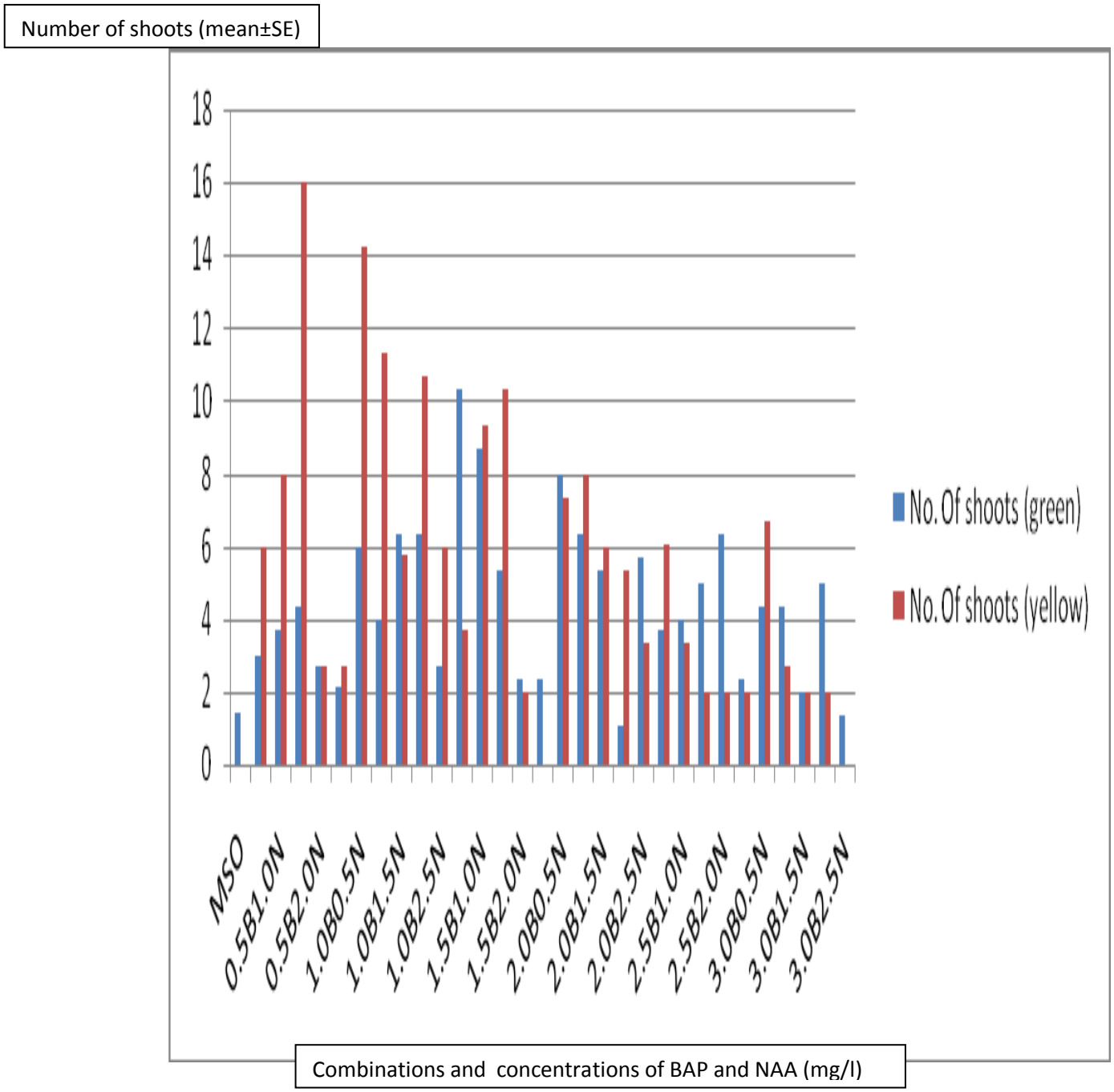


Figure 3.1: (a) Effects of BAP and NAA on shoot formation from the juvenile shoots (from green and yellow seeds) of *N. nucifera* after 24 weeks in culture on MS medium. The cultures were maintained at 25±1˚C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 24 weeks.

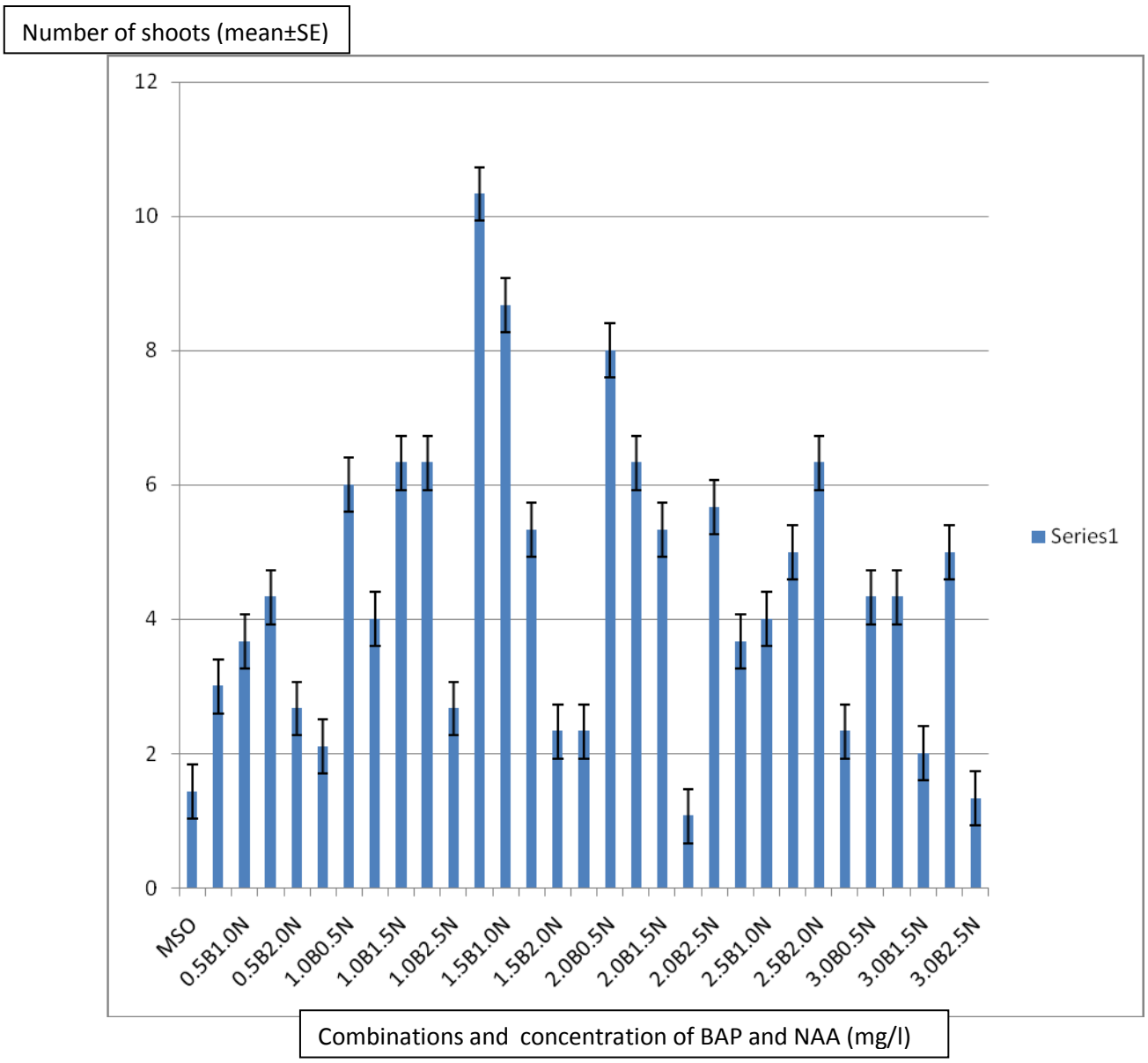


Figure 3.1: (b) Effects of BAP and NAA on shoot formation from the juvenile shoots (from green seeds) of *N. Nucifera* after 24 weeks in culture on MS medium. The cultures were maintained at 25±1˚C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 24 weeks.

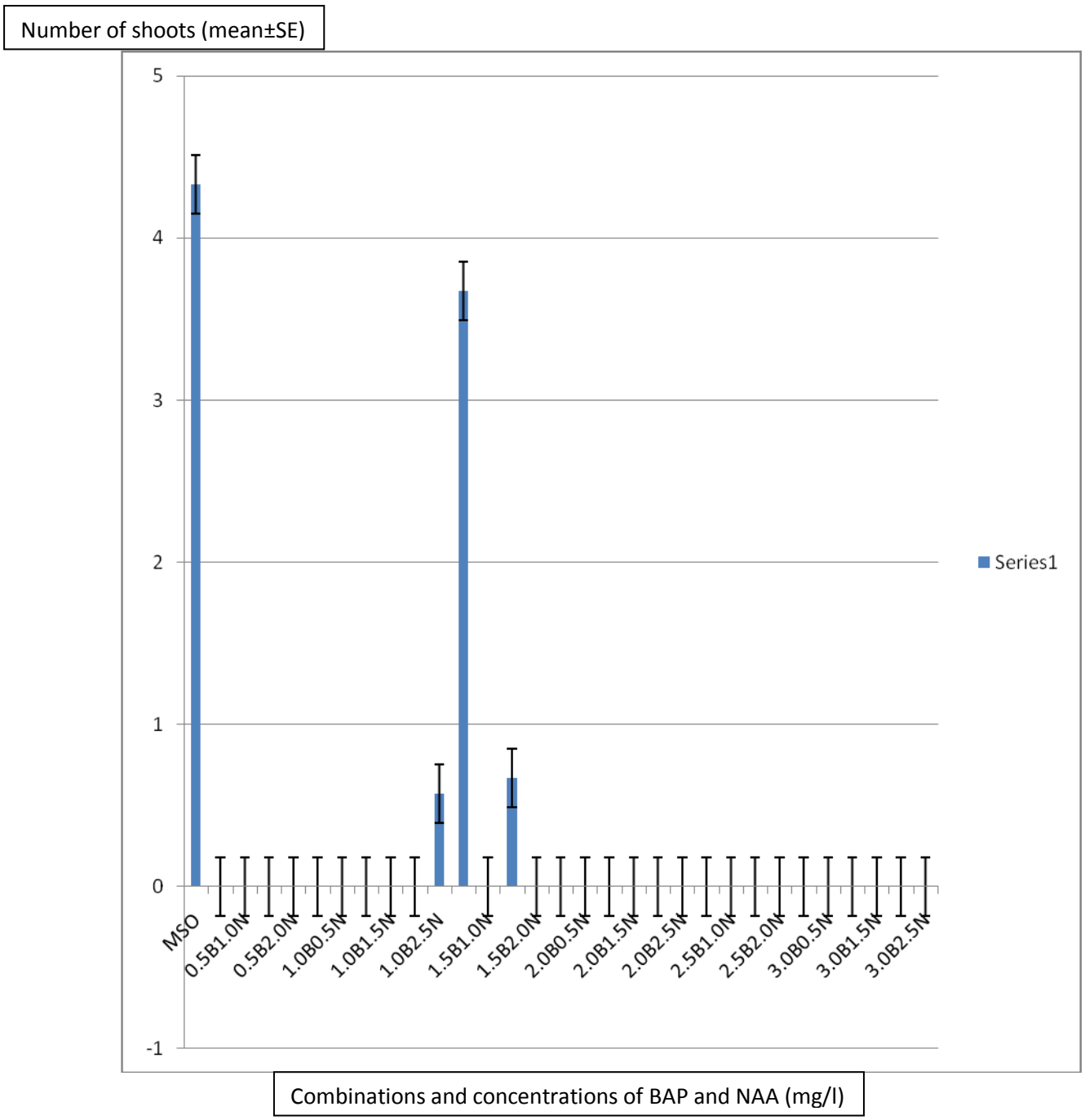


Figure 3.1: (c) Effects of BAP and NAA on root formation from the juvenile shoots (from green seeds) of *N. Nucifera* after 24 weeks in culture on MS medium. The cultures were maintained at 25±1˚C with 16 hours light and 8 hours dark , with 1000 lux intensity of light for 24 weeks.

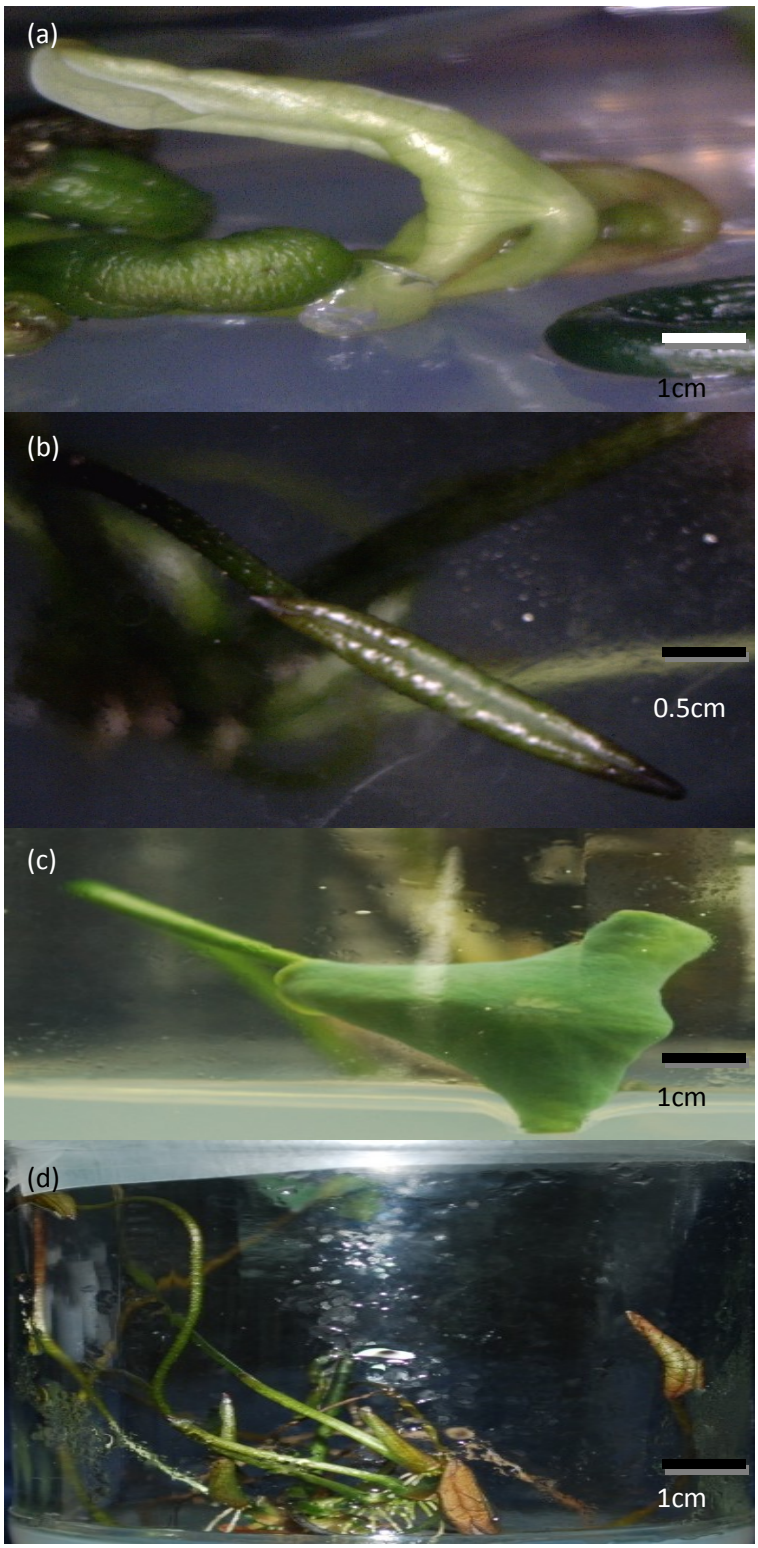


Figure 3.2: Green seeds cultured on MS solid media supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA, under 16 hrs light and 8 hrs dark, at 25±1^{o}C (a) Week 2, shoots elongation (b) Week 12, first rolled leaf (c) Week 16, first unrolled leaf (d)Week 24, primary roots formation with multiple shoots.

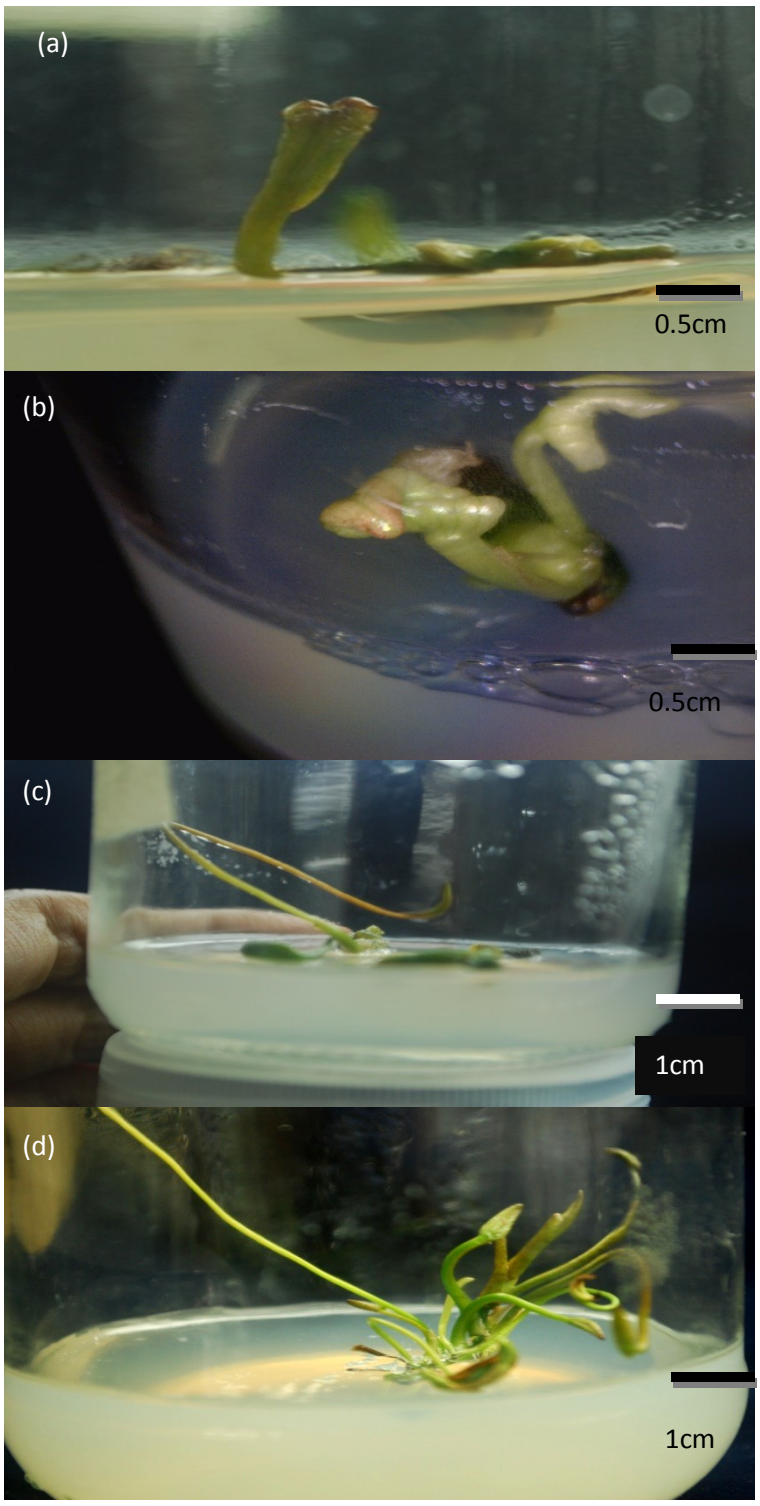


Figure 3.3: Yellow seeds cultured on MS solid media supplemented with 0.5 mg/l BAP and 1.5 mg/l NAA, under 16 hrs light and 8 hrs dark, at 25±1^{o}C (a) Shoots elongation after 2 week (b) First rolled leaf after 12 week (c) First unrolled leaf after 16 week (d) Layered shoots without root formation after 24 week.

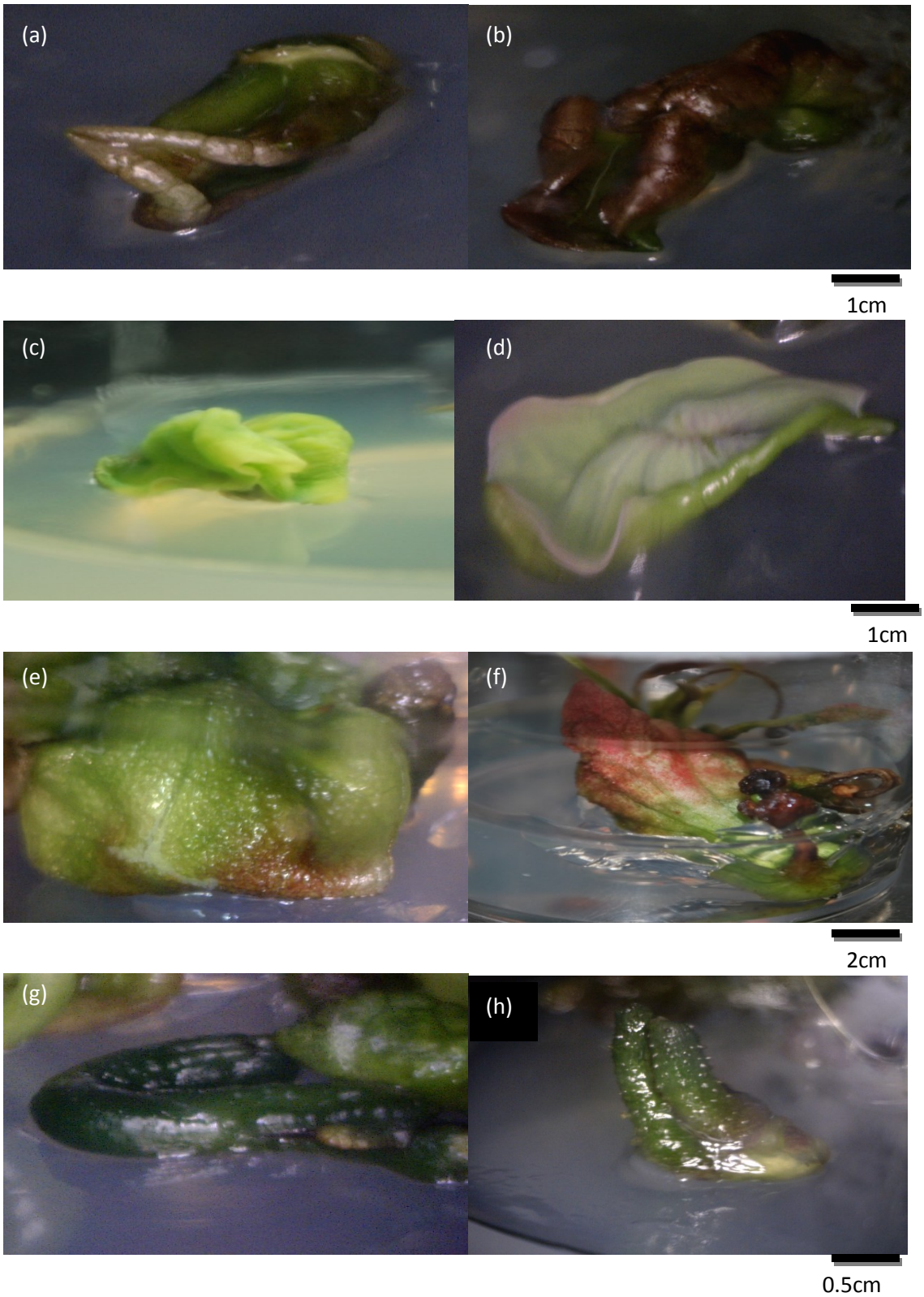


Figure 3.4: Abnormal leaf cultured on MS media supplemented with 1.5 mg/l BAP and 2.5 mg/l NAA after 10 weeks (a) Pink Shoots (b) red rolled leaf (c) Green spongy tissue (d) Green and round leaf (e) Pink and green oval leaf (f) Red and green oval leaf (g) Dark green stem (h) Stem with tiny thorns (prickles).

3.3.2 Determination of the Optimum MS Strength

Table 3.2 showed the response of i*n vitro* development of shoots in different strength of solid MS media. The results showed that explants from green seeds gave better response to higher MS strength. The highest shoots per explant for green seed explants was in 8.8mg/l MS powder (double strength) with mean 19.03±0.05 shoots per explant. This followed by 6.6mg/l MS powder (one and a half strength) with mean 16.01±10.82 shoots per explant. The lowest number of shoots per explants was in MS basal media with mean1.43 ±0.10 for green seed explants.

However, the highest shoots per explant was observed on 4.4 mg/l MS powder (full strength) was only for yellow seed explants with mean 16.06±0.06 shoots per explant. The lowest shoot per explant was observed from yellow seed explants which was on 6.6 mg/l (one and half strength) MS powder with 12.01±0.53 shoots per explant. No response of yellow seed explants on lower and higher MS strength. From this experiment, MS media with 4.4 mg/l (full strength) was selected as optimum for shoot formation in lotus from both green seed explants and yellow seeds explants.

Table 3.2 : *In vitro* development of shoots in different strength of MS media. Cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks.

MS Media Strength (mg/l)	No.of shoots per explant (Mean ±SE)		Observations
	Green Seeds	Yellow seeds	
0 MS (control)	1.43 ±0.10	0	Slow elongation of dark green shoots in week 1-4. yellow shoots turned to brown and necrosis better after 4 weeks
½ MS (2.2 mg/l)	4.00±0.15	0	Faster elongation of dark green shoots in week 1. yellow shoots turned to brown and necrosis better after 4 weeks
1 MS (4.4 mg/l)	10.33±0.23	**16.06±0.06**	Shoots elongation rapidly in week 3 for both type of explants
1 ½ MS (6.6 mg/l)	16.01±10.82	12.01±0.53	Shoots elongation for green seeds. While, yellow shoots turned to brown and necrosis better after 4 weeks
2 MS (8.8.mg/l)	**19.03±0.05**	0	Green shoots turned to yellowish in week 1. yellow shoots turned to brown and necrosis better after 4 weeks

Mean ± Standard error (SE), n=30. The bold numbers represent the best result.

3.3.3 Identification of the Optimum pH

Table 3.3 showed the effect of different pH media in regeneration of lotus shoots from juvenile shoots from green seed and yellow seed explants. Based on the results, pH range from 4.5-6.5 produced vigorous growth of regeneration of shoots for both green seed explant and yellow seeds. Media with pH 5.5 resulted in the highest height of shoots for green seed explants with mean **12.04±0.7** mm and **16.03±0.30 mm** for yellow seed explants. These were followed by MS media with pH 4.5 with mean 10.11±0.5 mm for green seed explants and 13.50±0.41 mm for yellow seed explants. The lowest were in pH 8.5 with mean 6.75±1.11 mm and 3.44±0.21 mm for green seed explants and yellow seed explants, respectively. Explants response better in acidic media compared to alkaline media. Therefore, pH 5.5 was suitable for both explants green and yellow seeds.

Table 3.3 : The effect of different pH in regeneration of shoots. Cultures were maintained on MS media supplemented with growth regulators (BAP+NAA) at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux light intensity for 4 weeks.

pH	Height of shoot,mm (Mean±SE)		Observations
	Green Seeds	Yellow Seeds	
4.5	10.11±0.5	13.50±0.41	Multiple green and yellow shoots elongation
5.5	**12.04±0.7**	**16.03±0.30**	**Multiple green shoots became thicker. Yellow shoots turned to green**
6.5	9.62±10.1	11.50±0.63	Multiple shoots elongation for both green and yellow seeds explants
7.5	7.21±0.22	8.93±0.71	Green shoots turned to yellow. Yellow shoots turned to brown.
8.5	6.75±1.11	3.44±0.21	Green shoots turned to yellow. Yellow shoots turned to brown.

Mean ± Standard error (SE), n=30.

3.3.4 Effects of Distance of the Light Source

Table 3.4 showed the effects of distance of the light source in multiplication of lotus shoots. The results showed that the highest height of shoots for green seed explants with 9.41±1.11 mm in 250.00 mm light distance. The lowest was in distance 50.00 mm with 1.21±1.01 mm height of shoots from green seed explants.

The highest height of shoots with 16.67±0.23 mm obtained from yellow seed explants in 200.00 mm light distance. The lowest was in 50.00 mm light distance with 9.12±0.51 mm height of shoots. All explants in the nearest light distance were dried and died. From this experiment, light distance of 200.00-250.00 mm was optimum for growth of shoots from green seed explants and 100.00-200.00 mm from yellow seed explants.

Table 3.4 : The effect of light distance in regeneration of rhoots. Cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks.

Light Distance (mm)	Height of shoot,mm (Mean ±SE)		Observations
	Green Seeds	Yellow Seeds	
0.0 (control)	0	0	Green and yellow shoots become dried and turned to brown
50.00	1.21±1.01	9.12±0.51	Wrinkle green shoots. Yellow shoots turned to green
100.00	7.67±0.09	10.67±0.09	Green shoots elongation. Yellow shoots turned to green
150.00	7.13±0.72	13.10±1.01	Green shoots elongation. Yellow shoots turned to green
200.00	9.00±0.15	**16.67±0.23**	Green and yellow shoots elongation
250.00	**9.41±1.11**	9.13±0.50	Green and yellow shoots elongation
300.00	4.67±0.09	6.33±0.18	Green and yellow shoots turned to brown

Mean ± Standard error (SE), n=30. The bold numbers represent the best result.

3.3.5 Root Formation on Solid and Liquid media

Table 3.5 showed the response and development of rooting of lotus shoots from green and yellow seeds when subcultured in rooting media. Shoots of about 15mm-25mm in height were detached from shoot clumps (Figure 3.6) and transferred to rooting media. Solid MS basal media was identified as the optimum rooting media for green seed explants with mean of 4.33±0.53 roots per explant (Figure 3.5 (b)) . However, the shoots were stunted with slow growth until maximum height of 30.00mm for 24 weeks (Figure 3.5 (a)). Slightly lower with 3.67 ±0.32 roots per explant for green seed explants on solid MS media supplemented with 1.5mg/l BAP and 0.5mg/l NAA. Roots formations on solid MS basal media were longer and thicker compared to solid MS media supplemented with BAP and NAA.

Unfortunately, no roots formation from yellow seed explants. Alternatively, layered multiple shoots were transferred to solid MS media after 24 weeks on solid MS media supplemented with 0.5mg/l BAP and 1.5mg/l NAA (Figure 3.7). Within 4 weeks, only primary roots occurred, while, for green seed explants, primary and secondary roots occurred.

Table 3.5 : The effect of root induction on solid and liquid media. Cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks.

MS medium	No of roots per explant (Mean ±SE)	
	green seeds	yellow seeds
Solid MS basal (control)	4.33±0.53	0
Liquid MS basal	0	0
Solid MS+1.5mg/lBAP+0.5mg/lNAA	3.67 ±0.32	0
Liquid MS+1.5mg/lBAP+0.5mg/lNAA	0	0
Solid MS+0.5mg/lBAP+1.5mg/lNAA	0	0
Transfer to solid MS basal media (after 24 weeks on Solid MS+1.5mg/lBAP+0.5mg/lNAA)	Multiple roots* (primary and secondary roots)	Multiple roots* (primary roots only)
Transfer to solid MS basal media (after 24 weeks on Solid MS+0.5mg/lBAP+1.5mg/lNAA)	Multiple roots* (primary and secondary roots)	Multiple roots* (primary roots only)

*Multiple roots = more than 20 roots.
Mean ± Standard error (SE), n=30.

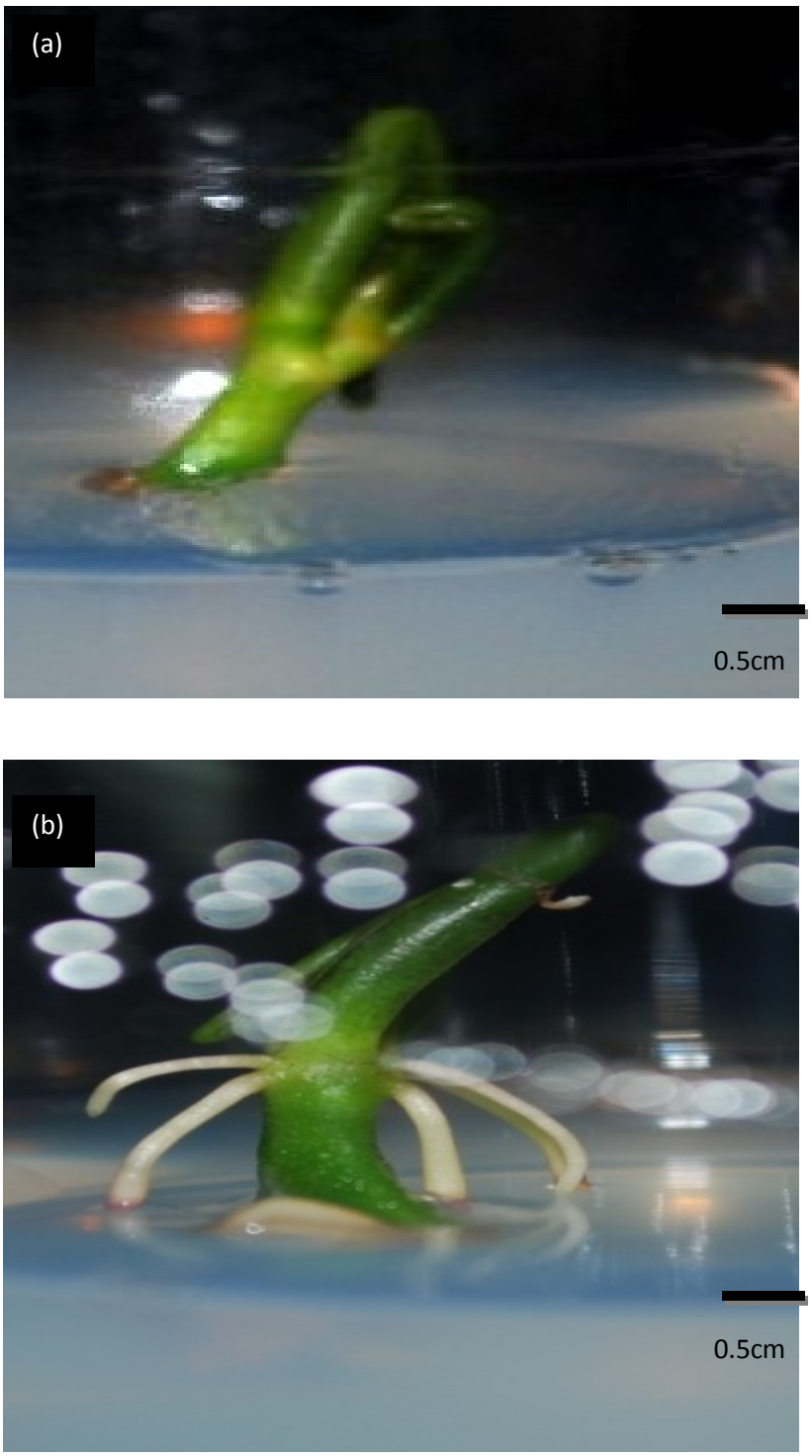


Figure 3.5: Rooting of coatless green seed explants on solid MS basal media (a) Dark green rolled plumules (b) After 24 weeks (6 months), explants with the same characteristic of rolled plumules but additional with 5 white primary roots (pink at the root tips).

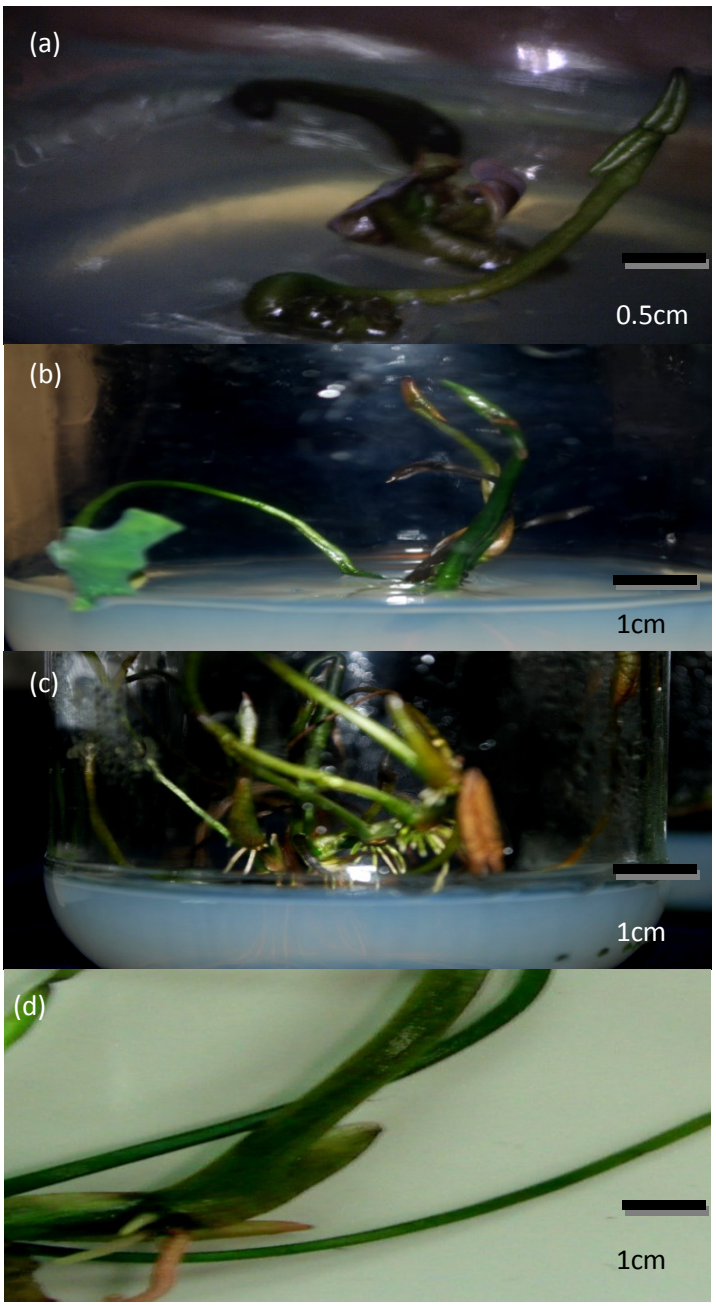


Figure 3.6: Subculture of 2-week-old green seed explants on solid MS media supplemented with 1.5mg/l BAP and 0.5mg/l NAA (a) 4-week-old; shoots elongation (b) 16-week-old; 3 rolled leaf and 1 unrolled leaf (c) 24-week-old; primary roots formation (d) Internode characteristic; 2 pink and 2 white primary roots.

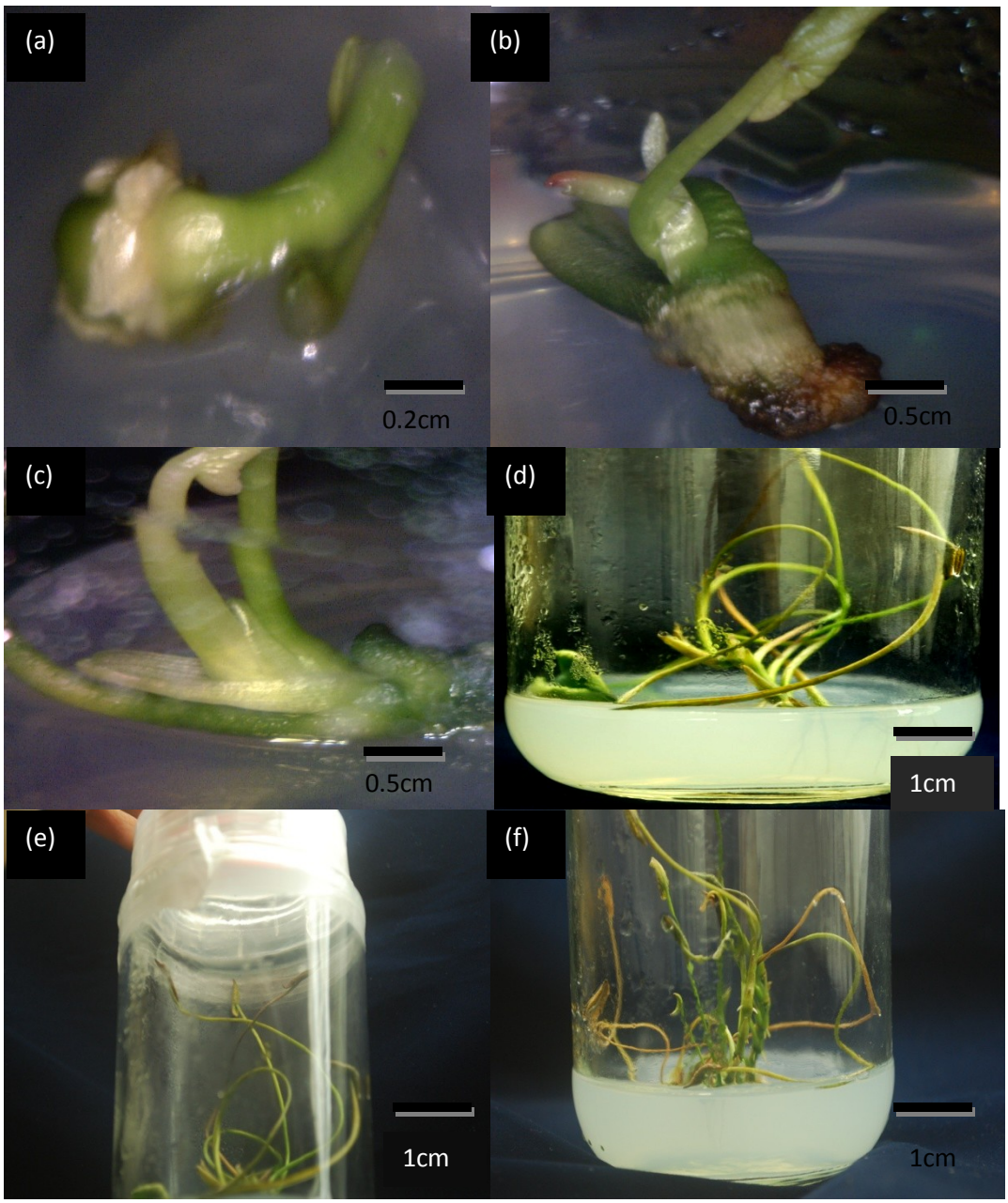


Figure 3.7: 2-week-old yellow seed explants subcultured on solid MS media supplemented with 0.5mg/l BAP and 1.5mg/l NAA (a) 2-week-old yellow seed explants (b) 4-week-old elongated shoots (c) 6-week-old layered shoots (d) layered shoots elongation after 12 weeks (e) 14-week-old rolled leaf (f) 24-week-old primary root formation.

3.3.6 Effects of Double-layer of Liquid and Solid Media

Table 3.6 showed the effects of solid and liquid media at different level in regeneration of explants from green and yellow seeds. As control (Figure 3.8(a)), explants were cultured on solid MS basal media. Green shoots were elongated with mean of 4.31±0.80 number of shoots per explant and formation of primary root (Figure 3.8(b)). While, yellow shoots turned to green with mean number of 9.10±0.51 shoots per explant and formation of primary roots. The highest number of shoots per explant was both in ratio liquid to solid 2:1 with mean number of 16.67±0.23 shoots per explant with formation of primary and secondary roots for explants from yellow seeds (Figure 3.8(c)), while mean 9.00±0.15 number of shoots per explant for green seeds with formation of layered multiple shoots. In ratio liquid to solid with 1:1, both shoots elongated normally with mean 8.33±0.23 and 15.67±0.09 number of shoots per explant for green and yellow shoots, respectively (Figure 3.8(d)). Even though lotus is an aquatic plant, in ratio liquid to solid 3:1 (flooded), both shoots turned to brown with mean number of 5.33±0.23 shoots per explant for green shoots and 10.33±0.23 shoots per explant for yellow shoots.

Figure 3.9 showed the best explants (juvenile shoots excised from yellow seeds) regenerated on optimum solid MS media supplemented with 0.5 mg/l BAP and 1.5 mg/l NAA for 20 weeks, developed with new characteristic (layered multiple shoots).

Table 3.6 : The Effect of Solid level Media in Regeneration of Shoots. Cultures were maintained at 25±1°C with 16 hours light and 8 hours dark, with 1000 lux intensity of light for 4 weeks.

Ratio liquid:solid (mm)	Explant	No. of shoots per explant (Mean ±SE)	Observations
0:1 (control)	Green shoot	4.31±0.80	Dark green shoots elongated. Primary root formation.
	Yellow shoot	9.10±0.51	Yellow shoots turned to green and elongated. Primary root formation.
1:1	Green shoot	8.33±0.23	Shoots elongated
	Yellow shoot	15.67±0.09	Shoots elongated
2:1	Green shoot	**9.00±0.15**	Shoots creeping and formed rolled leaf**.** Primary and secondary root formation
	Yellow shoot	**16.67±0.23**	Shoots over layered. Primary root formation
3:1	Green shoot	5.33±0.23	Shoots turned to brown
	Yellow shoot	10.33±0.23	Shoots turned to brown

*Double layer medium (green seed): combination of Solid MS+1.5 mg/l BAP +0.5 mg/l NAA and liquid MS basal

*Double layer medium (yellow seed): combination of Solid MS+0.5 mg/l BAP +1.5 mg/l NAA and liquid MS basal

Mean ± Standard error (SE), n=30. The bold numbers represent the best result.

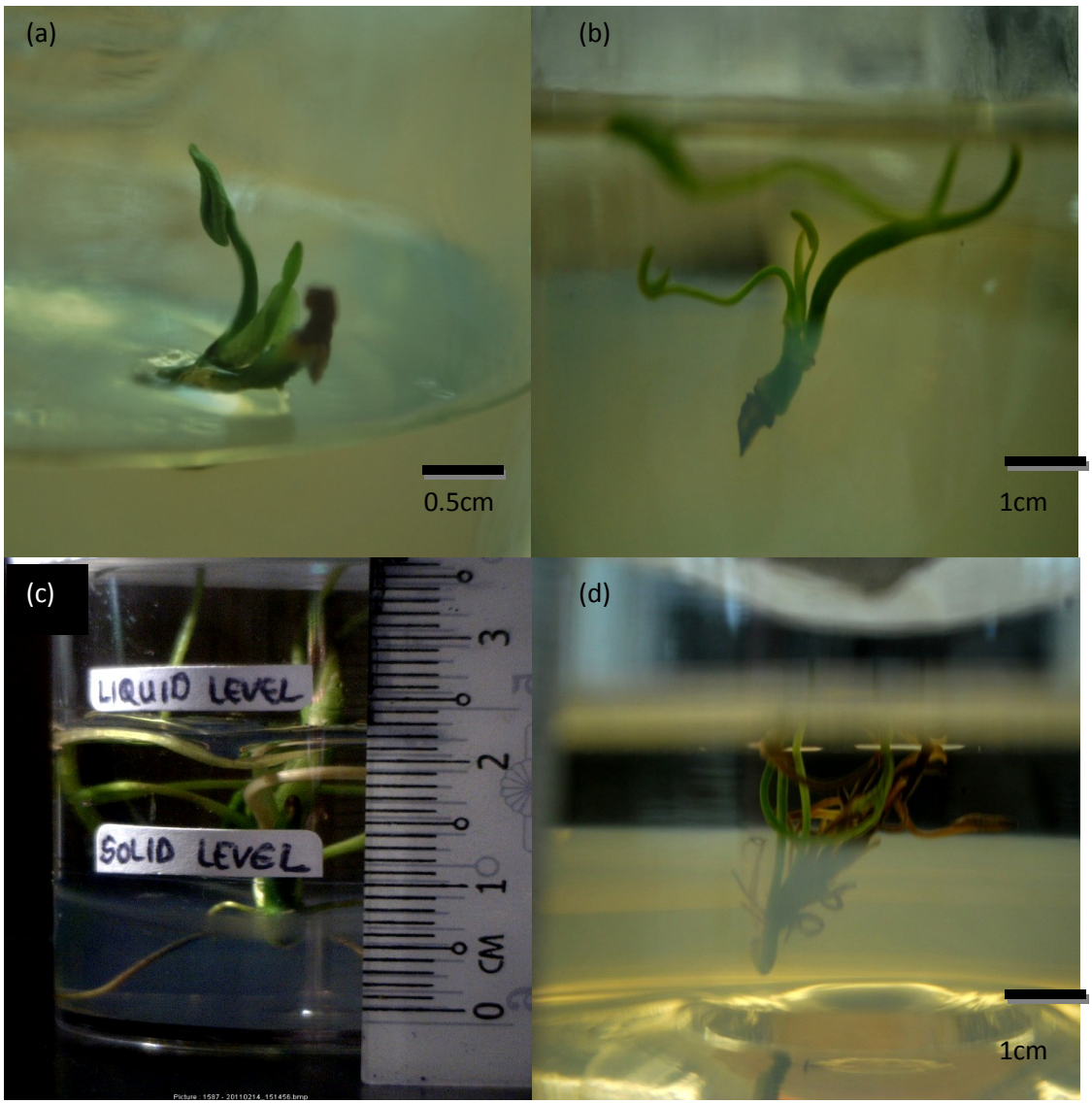


Figure 3.8: Effects of solid (with combination BAP and NAA) and liquid (MS basal) media on juvenile explants (a) Shoots without root elongated on solid MS media and need to subculture or transfer every 21 days to avoid necrosis (b) Shoots without root elongated on solid MS media added with liquid MS basal (c) Plantlet maintained in double layer media (without transferring for up to 10 months) (d) Rooting from yellow seeds with shoots elongation on solid MS media supplemented with 0.5 mg/l BAP and 1.5 mg/l NAA (base) and liquid MS basal media (top).

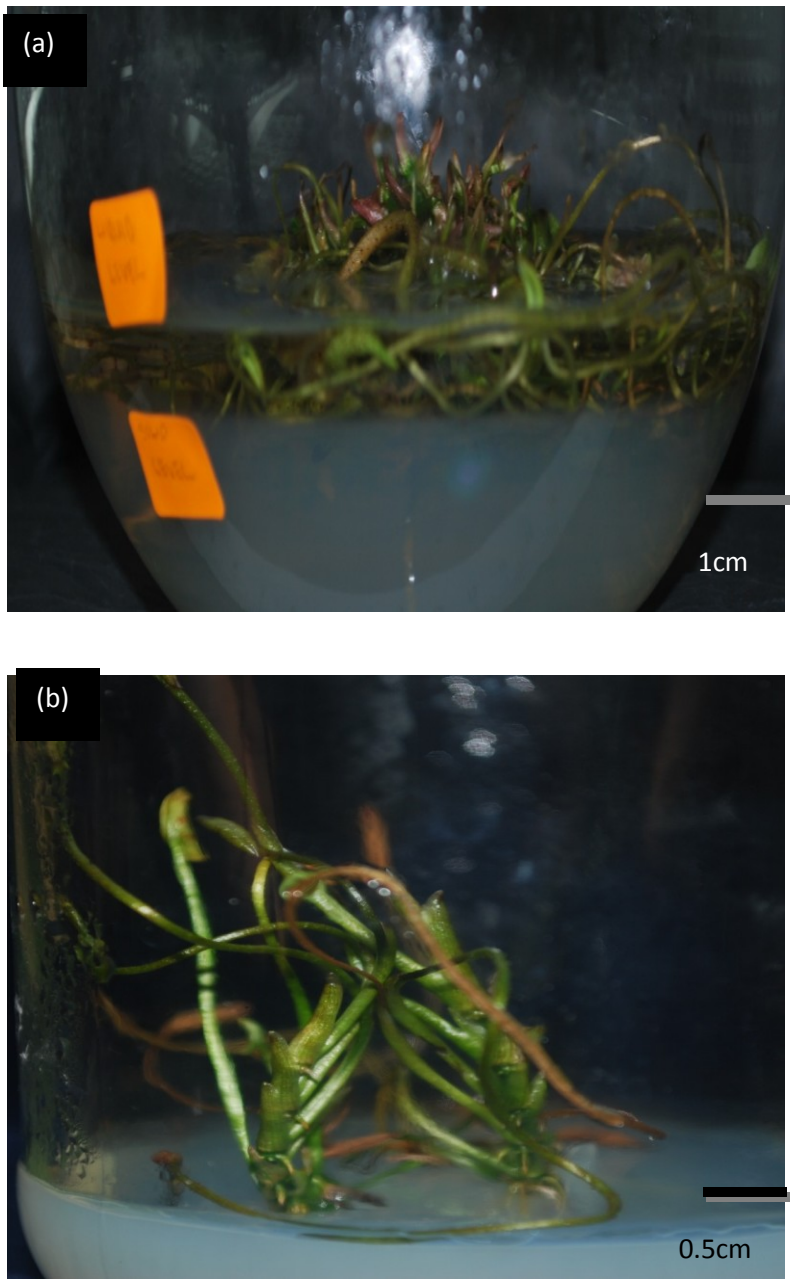


Figure 3.9 : Regeneration of yellow seed explants (a) 10-month-old vigorous shoots elongation in double layer media (without root) (b)The best explants (juvenile shoot excised from yellow seed) regenerated on optimum solid MS media supplemented with 0.5 mg/l BAP and 1.5 mg/l NAA for 20 weeks, developed with new characteristic (layered multiple shoots). Then, transferred 4 weeks to MS solid media for roots formation. The cultures were maintained at 25±1˚C with 16 hours light and 8 hours dark, with 1000 lux light intensity for 24 weeks.

3.4 SUMMARY OF RESULTS

1. Direct plant regeneration of *Nelumbo nucifera* Gaertn. were successfully achieved from green seed explants cultured on solid MS media supplemented with combinations of 1.5 mg/l BAP and 0.5 mg/l NAA with **10.33 ±0.23** shoots per explants and also completed with 3.67 ±0 roots per explant.

2. Direct regeneration of *Nelumbo nucifera* Gaertn. was also successfully achieved from yellow seed explants cultured on solid MS media supplemented with combinations of 0.5 mg/l BAP and 1.5 mg/l NAA with **16.00±0.30** shoots per explants, with new leaf characteristic of layered multiple shoots. Roots formed on solid MS basal media with 4.33±0.53 roots per explant.

3. Some formation of abnormal shoots (pinkish, red and oval leaf) occurred from green seed explants on solid MS media supplemented with combinations of BAP and NAA; 1.0 mg/l BAP and 2.5 mg/l NAA, 2.5 mg/l BAP and 2.5 mg/l NAA, 1.5 mg/l BAP and 2.0 mg/l NAA , 1.5 mg/l BAP and 2.5 mg/l NAA.

4. The highest shoots per explant for green seed explants was obtained in 8.8mg/l MS powder (double strength) with mean of **19.03±0.05** shoots per explant.

5. The highest shoots per explant for yellow seed explants was on 4.4 mg/l MS powder (full strength) with mean of **16.06±0.06** shoots per explant.

6. Media with pH 5.5 resulted in the highest height of shoots for green seed explants with mean of **12.04±0.7** mm and **16.03±0.30 mm** for yellow seed explants.

7. The highest shoots height for green seed explants with **9.41±1.11 mm** obtained in media placed at 250.00mm light distance.

8. The highest shoot height with **16.67±0.23 mm** for yellow seed explants obtained in media 200.00 mm light distance.

9. Solid MS basal media was optimum for root formation within 4 weeks for both green seed explants and yellow seed explants after 24 weeks on solid MS media supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA.

10. The highest number of shoots per explant was both observed in ratio liquid to solid 2:1 with mean of 16.67±0.23 number of shoots per explant with formation of primary and secondary roots from explants from yellow seeds, while mean 9.00±0.15 number of shoots per explant for green seeds with formation of layered multiple shoots.

CHAPTER 4

PIGMENT DETECTION AND EXTRACTION

in *Nelumbo nucifera* Gaertn.

4.1 EXPERIMENTAL AIMS

Nelumbo nucifera Gaertn. (pink Asian lotus), a perennial aquatic plant that also known as one of the medicinally versatile plants in traditional medical practices. Natural pigment extracts from medicinal plants are not associated with side effects and have enormous therapeutic potential to heal many infectious diseases and can be developed as a better new drugs against microbial infection (Benkeblia, 2004).

Natural pigments or colourants in plants arise from two main classes of pigments, carotenoids and anthocyanins. Carotenoids are one of the largest classes of natural pigments synthesized in all photosynthetic organisms (plants, algae and cyanobacteria) and in some non-photosynthetic organisms such as bacteria and fungi (Burkhardt et al., 1997). Mammals including humans cannot synthesize carotenoids even though they are essential source of retinoids and vitamin A (Botella-Pavia et al., 2004) and are responsible for the colour of familiar animals such as lobster, flamingo and fish (Klaui and Bauernfeind, 1981). In plants the carotenoid pigments are synthesized in the plastids. Carotenoids are responsible for the orange and yellow lipid soluble pigments in plastids (Van den Berg et al., 2000; Tevini et al., 1984).

Carotenoids are found in algae, fungi, yeasts, moulds, mushrooms, bacteria and in all classes of plants and animals (Chandrika, 2009). The typical carotenoids found in plant chloroplasts are lutein, zeaxanthin, antheroxanthin, violaxanthin and neoxanthin and in chromoplasts are capsanthin, capsorubin, bixin, crocetin and citraurin (Van den Berg et al., 2000).

The sources for yellow dyes are enormous and derived either from flavonoid or carotenoid (Melo, 2009). Contrary to the red or blue dyes, yellow dyes are considerably less resistant to fading (Ferreira et al., 2004 and Bohmer, 2002). Table 4.1 indicates the functions of carotenoids. The most commonly used natural carotenoids colorants in food are extracted from *Bixa orellana* (Bixin), *Capsicum annuum* (Beta-carotene, capsanthin), *Crocus sativus* (crocetin), *Tagetes erecta* (lutein, zeaxanthin), *Daucus carota* (beta-carotene) or *Lycopersicon esculentum* (lycopene) (Chandrika, 2009).

Table 4.1: Functions of carotenoids.

Functions	Carotenoids	Reference:
Provitamin A activity	β-carotene, α-carotene, β-cryptoxanthin	Van Vliet et al. (1996)
Antioxidant	all carotenoids	Palozza and Krinsky (1992)
Cell communication (Morphogenesis and cell differentiation)	β-carotene, canthaxanthin, cryptoxanthin	Stahl and Siess (1998)
Immune function enhancers	β-carotene	Solomons and Bulux (1997)
UV skin protectant	β-carotene, lycopene	Lindley (1998)
Macula protection	lutein, zeaxanthin	Seddon et al. (1994)

Pharmacological studies of lotus plant revealed that the whole parts posses antidiabetic, antypyretic, antiinflammatory, anticancerous, antimicrobial, antiviral and

anti-obesity properties (Kashiwada et al., 2005). The major constituents isolated from lotus plant are alkaloids (liensinine, nuciferine, remrefidine and isoliensinine) and flavonoids ((+)-1(R)-coclaurine, (-)-1(S)-norcoclaurine and quercetin 3-O-β-D-glucuronide) (Sridhar and Bhat, 2007). Previous studies reported that betulinic acid isolated from lotus rhizomes used as anti-tumor and melanoma specific cytotoxic agent (Mukherjee et al., 1997). Lotus seeds would suppress cell cycle progression, cytokine genes expression and cell proliferation in human peripheral blood mononuclear cells (Bensky et al., 2004). Lotus leaves showed the hypotensive effects that mediated by vasodilation via nitric oxide (Tongtorsak et al., 2004). Recently, lotus flower is shown to have high potential in treatment of various ailments including cancer, hypertention, diarrhea, fever, weakness, infection and body heat imbalance (Saengkhae et al., 2008).

The aim of this research is to explore the potential of natural colorants from lotus stamen (*in vivo*) for industry and lotus leaves (*in vivo* and *in vitro*) for medicinal purposes. To achieve this, three main objectives have been defined:

1. to establish analytical method for carotenoid analysis through HPLC system
2. to investigate the potential of carotenoid composition for commercial applications such as coating as natural colorants.
3. to study microbial activity of lotus rolled leaves grown *in vivo* and *in vitro*.

4.2 MATERIALS AND METHODS

4.2.1 Plant Sample Preparation

Yellow stamens of lotus collected from Tasik Chini in Pahang , Malaysia were freeze dried for 72 hours, after which the samples were ground into fine powder and stored at -20°C until further analysis.

4.2.2 Pigment Preparation

1.0 g of powdered freeze-dried material was extracted with 10 ml of acetone to allow efficient solvent penetration. The solution was then allowed to stand overnight in darkness at room temperature ($25^{o}C\pm1$). The following day the samples were vortexed and centrifuged for 2 minutes at 13 500 *g* and the supernatant transferred to a foil covered 50 ml graduated polypropylene centrifuge tube.

4.2.3 Effects of pH on Carotenoid Stability

The yellow supernatant from pigment preparation was tested with addition of HCl and NaOH. pH were measured using pH meter and maintained at pH 2, 4, 6, 8, 10, 12 and 14. Colour changed were observed. Three replicates for each treatment were used.

4.2.4 Carotenoid Extraction and Analysis Through HPLC System

4.2.4.1 Extraction of carotenoids

The extraction procedure essentially followed the methods described by Morris et al. (2004) and Othman (2009). For each sample, 1.0 g of powdered freeze-dried material was rehydrated by adding 1 ml of distilled water, followed by 5 ml of acetone. The solution was then allowed to stand overnight in darkness at room temperature. The following day the samples were vortexed and centrifuged for 2 minutes at 13 500 *g* and the supernatant transferred to a foil covered 50 ml graduated polypropylene centrifuge tube and were stored at 4 ºC in the dark prior to analysis.

4.2.4.2 Data Analysis

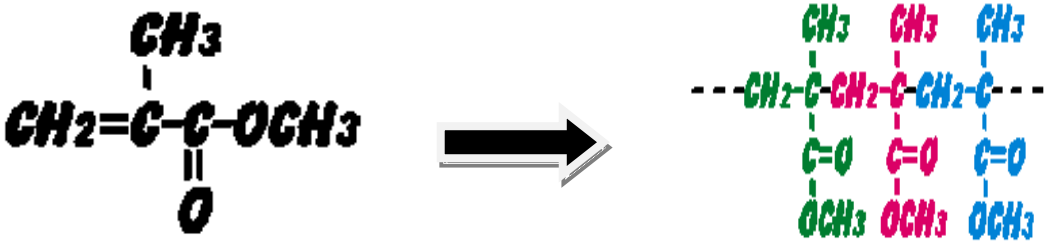
Total carotenoid concentration was determined by spectrophotometry and was measured at three different wavelengths: λ 480 nm, 648 nm and 666 nm using Shimadzu UV-1650 PC spectrophotometer as described by Britton et al. (1995) and Othman (2009). Individual carotenoid concentration was determined by reverse phase HPLC on an Agilent model 2100 series comprised of a binary pump with autosampler injector, micro vacuum degassers, thermostatted column compartment and a diode array detector as detailed in Othman (2009). The column used was a Luna C_{18} end capped 5 µm, 250 x 4.6 mm reverse phase column (Agilent, Malaysia). The solvents used were (A) acetonitrile: water (9:1 v/v) and (B) ethyl acetate. The solvent gradient used developed as follows: 0-40% solvent B (0-20 min), 40-60% solvent B (20-25 min), 60-100% solvent B (25-25.1 min), 100% solvent B (25.1-35 min) and 100-0% solvent B (35-35.1 min) at a flow rate of 1.0 ml min^{-1}. The column was allowed to re-equilibrate

in 100% solvent A for 10 min prior to the next injection. The temperature of the column was maintained at 20ºC. The injection volume was 10 µL. Carotenoid standards β-carotene, violaxanthin, lutein, zeaxanthin and neoxanthin were obtained commercially from Sigma-Aldrich.

4.2.5 Carotenoid as Natural Coating

4.2.5.1 Resin Preparation and Coating

80ml tetrahydrofuran (THF) solvent, 20g acrylicpolyol (binder) and 20g of polymethylmetacrylate powder (PMMA) was heated at 50^{o}C until the powder dissolved. The resin was cooled at room temperature before use. 5ml of 20% PMMA was mixed with 5ml of pigment. The pigment-resin solution 1:1 was coated onto glass slides using paint brush.



Methyl Methacrylate (MMA) Acrylic resin (PMMA)

4.2.5.2 Effect of Cobinder

The pigment-resin solution was added to 1% acid as cobinder for stability purposes. Pigment-resin solution with optimum cobinder will avoid fadius for the coating.

4.2.5.3 Data Analysis

Measurement of carotenoid density was determined using Photometre Shimadzu UV-1650 PC. Glossiness of coatings were measured using glossimetre (standard 91.6^{o}).

4.2.6 Chlorophyl Extraction and Microbial Activity

Medicinal and herbal plants which contain components of therapeutic have been used as remedies for human diseases for centuries. Plants can produce antifungal compounds from leaf extraction to protect themselves from biotic attack that could be esential for fungi infection resistance (Wotjaszek, 1997). Plant also are rich in a wide variety of secondary metabolites polyphenols, such as tannins, terpenoids, alkaloids, and flavonoids, which have been demonstrated to have *in vitro* antimicrobial properties (Gonzalez-Lamothe, et al.,2009). The variety of compounds produced in *in vivo* and *in vitro* plants can show different bioactivity potentials and this is similar with the bioactivities, it will differ between *in vitro* and *in vivo* grown plants. Antimicrobial activity differ *in vivo* and *in vitro*, probably due to the inherent characteristics of the fully grown plants and the maturity of its chemically active constituents.

Present study is to investigate antimicrobial properties in lotus rolled leaves, *in vivo* and *in vitro* . Until to date, this is the first study on comparison of the antimicrobial properties of *in vivo* and *in vitro* grown *lotus.*

4.2.6.1 *In vivo* plant samples

Rolled leaves of *Nelumbo nucifera* Gaertn.were collected from Rimba Ilmu in University of Malaya, Kuala Lumpur, Malaysia.

4.2.6.2 *In vitro* plant samples

Green seeds (fruits) were surface sterilized by soaking in 99% (v/v) chlorox solution and 70% (v/v) ethanol for 2 minutes. The sterilized seeds were then germinated on solid Murashige and Skoog basal media (Murashige and Skoog, 1962). 4-week-old rolled shoots were used for hydroethanolic extraction.

4.2.6.3 Test Organisms

The antimicrobial screening organisms consists of *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillis cereus*, *Escherichia coli*, *Aspergillus niger*, *Trichoderma* species and *Fusarium* species. All species of microbes were obtained from microbial laboratory at Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia.

4.2.6.4 Sample extraction

Six months old fresh sample of rolled leaves (2 g) which were in 1 cm diameter from both *in vivo* and *in vitro* were ground using mortar. 20ml of 90% (v/v) ethanol was added then allowed to stand overnight in the dark at room temperature. The following day, the samples were centrifuged for 2 minutes at 13 500 *g* and the supernatant transferred to a foil covered 50 ml graduated polypropylene centrifuge tube and were stored at 4 ºC in the dark prior to analysis. The solution were evaporated by using rotary evaporator at 40±1^{o}C for 30 minutes. The plant extracts were dissolved in phosphate buffered saline (PBS) and kept until required for the subsequent experiments.

4.2.6.5 Antimicrobial test (paper disk diffusion method)

0.1 gram test bacteria were streaked on bacto agar medium plates, while fungi and yeast were streaked on potato Dextrose Agar (PDA) by using hockey steak. Sterilized filter paper disc (6mm) were soaked in hydroethanolic extracts (10mg/ml) and were then placed in the centre of test microorganisms. The plates were incubated for 48 hours and the diameters of the inhibition zones were measured. Tetracycline and antibiotic chloramphenicole (15µg/ml) disc were used as the positive controls. Experiments were done in three replicates.

4.3 RESULTS

4.3.1 Effect of pH on Carotenoid Colour

Figure 4.1 (a) showed 100g fresh yellow stamens of lotus collected from Tasik Chini, Pahang used to prepare 10g of dried ground sample (Figure 4.1 (b)). Bright and clear yellow supernatant in acetone solution (Figure 4.1 (e)) was used to stain the cotton (Figure 4.1 (c)). Dried residue with grey colour (Figure 4.1 (d)).

Table 4.1 and Figure 4.2 showed the effects of different pH on bright and clear yellow supernatant in acetone solution of lotus stamen. At acidic condition (pH 1-6), yellow pigment changed to the darker colour, yellow-brown. While, at strong alkaline condition (pH 12-14), the colour changed to yellow-green. The optimum yellow colour was at pH 8-10.

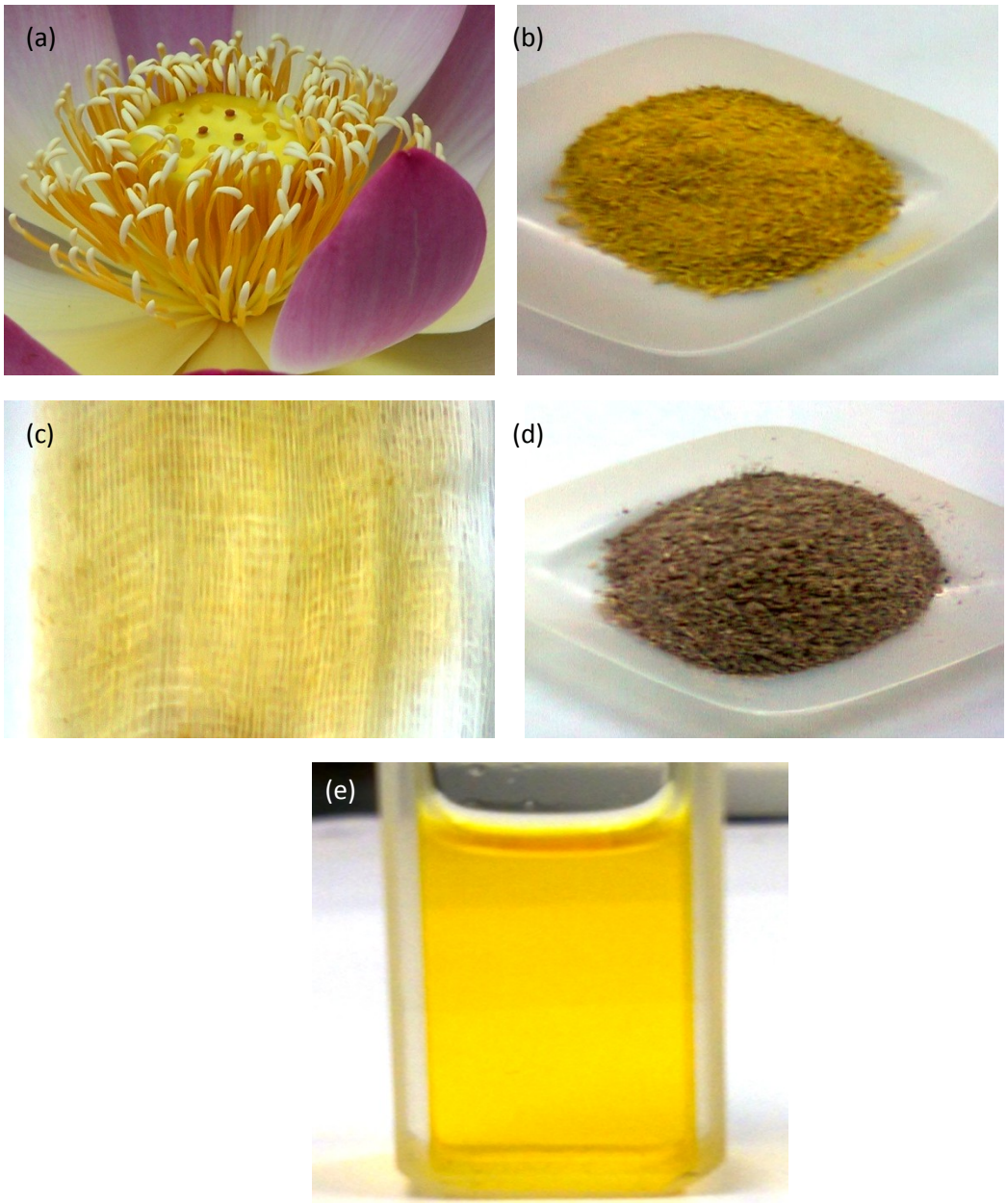


Figure 4.1 : Source of carotenoids in lotus (a) fresh yellow stamens of lotus collected from Tasik Chini, Pahang (b)dried ground yellow stamen (c) yellow stained of lotus on cotton (d) dried grey residue (e) Bright and clear yellow supernatant in acetone solution.

Table 4.1 : Effects of pH on yellow pigment (carotenoid) from lotus stamen.

pH	Colour
2	Yellow-brown
4	Yellow-brown
6	Yellow-brown
8	Yellow-orange
10	Yellow-orange
12	Yellow-green
14	Yellow-green

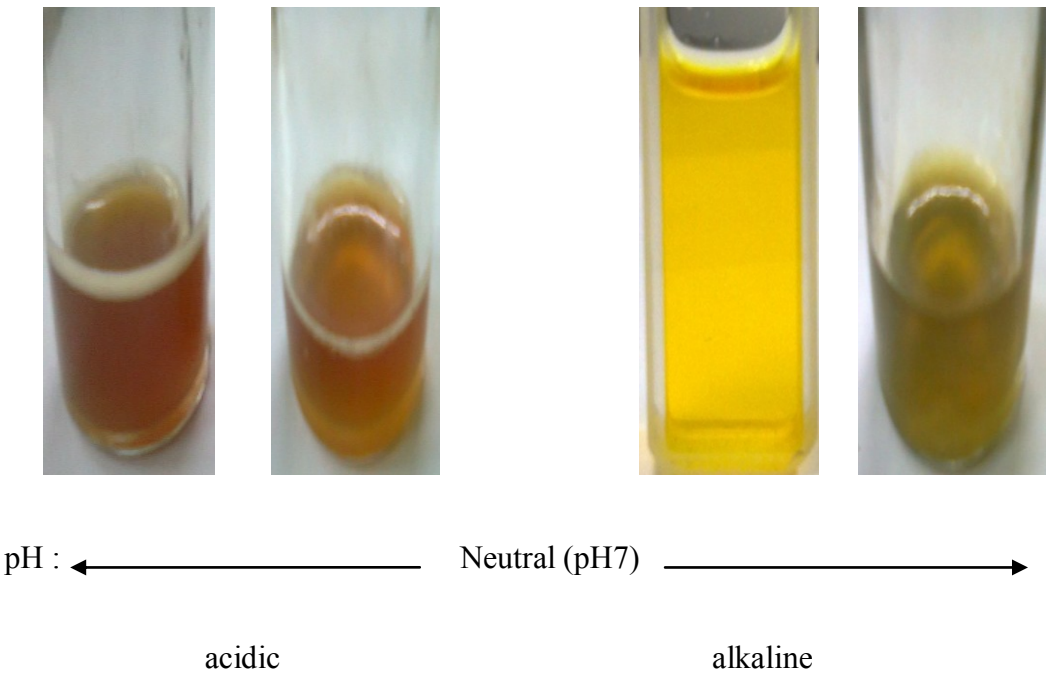


Figure 4.2 : Yellow pigment changed to yellow-brown with range of pH 1-6. Original at pH 8-10. While, range of pH 12-14 with yellow-green colour.

4.3.2 Analysis of total and individual carotenoid content in lotus flower stamen

Table 4.2 showed that the highest total carotenoid content (258.95 μg/g DW) was detected in sample 1 and in contrast, the lowest total carotenoid concentration was found in sample 2 (116.29 μg/g DW). Carotenoid analysis performed by HPLC system detected at least three major carotenoid peaks: neoxanthin, unknown and β-carotene (Figure 4.3). The ranges in content for all individual carotenoid pigments among all samples studied were shown in Table 4.2. As shown in Figure 4.3 and Table 4.2, beta-carotene was found substantially higher compared to neoxanthin and unknown carotenoid. Violaxanthin, zeaxanthin and lutein were not found in any of the samples analysed. The Wellburn Equation (Wellburn, 1994), in chloroform was applied to obtain the total carotenoid content as described below:

$$C_a = 10.91A_{666} - 1.2A_{648}$$

$$C_b = 16.36A_{648} - 4.57A_{666}$$

$$C_{x+c} = (1000A_{480} - 1.42C_a - 46.09C_b)/202 \ (\mu g/ml)$$

Table 4.2: Total and individual carotenoid content (µg/g DW) of stamen tissue of lotus flower from Tasik Chini, Pahang, Malaysia.

Sample	Neoxanthin (µg/g DW)	Violaxanthin (µg/g DW)	Zeaxanthin (µg/g DW)	Lutein (µg/g DW)	β-Carotene (µg/g DW)	Total Carotenoid (µg/g DW)
1	30.29	ND	ND	ND	228.01	258.95
2	20.90	ND	ND	ND	113.10	134.28
3	13.01	ND	ND	ND	102.96	116.29

ND-Non-detectable

DW-dried weight of sample/extract

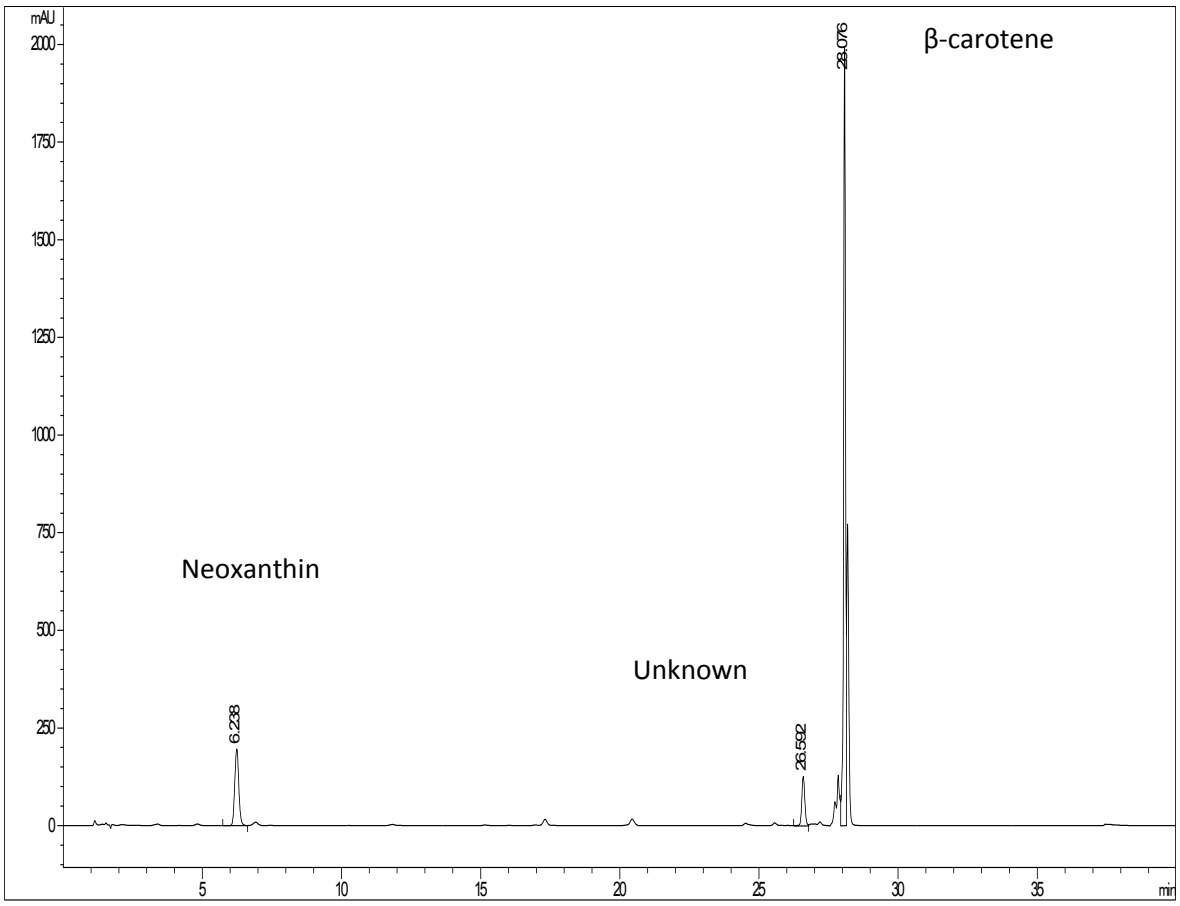


Figure 4.3: HPLC analysis of individual carotenoid of lotus flower (stamen and stigma). Chromatography, absorption and retention time of lotus stamen carotenoid content and composition (6.2 minute – neoxanthin, 26.6 minutes – unknown carotenoid, 28.1 minutes – beta-carotene).

Analysis of total and individual carotenoid content of lotus flower (stamen and stigma)

Lotus flowers were found to have the total carotenoid content of 526.96 ± 0.52 µg/g DW whereas for individual Carotenoid β-carotene (460 ± 10.28 µg/g DW) was found with a relatively high concentration and neoxanthin (39.26 ± 0.82 µg/g DW) was found in lower concentrations. One unknown Carotenoid also was detected.

4.3.3 Coating from Lotus Stamen

Table 4.3 showed the ratio of dried stamen sample to volume of 70% (v/v) acetone solution. The best bright yellow colour was from 1.0g/20.0ml (w/v) extraction. In 1.0g/10.0ml (w/v) extraction, the stamen sample was not dissolved. While in 1.0g/30.0ml (w/v) extraction, the yellow colour was not too strong.

Table 4.4 showed the glossiness and ratio of pigment and resin in coating process. The highest glosiness was in 1.0ml/30.0ml (v/v) of pigment-resin solution with mean of 74.67±0.33 GU. These followed by 1.0ml/20.0ml (v/v) of pigment-resin solution with mean of 67.01±0.58 GU. The lowest glosiness was in 1.0ml/10.0ml (v/v) of pigment-resin solution with mean of 57.67±0.67 GU. However, better bright red colour with smooth coating only occurred in the lowest glosiness pigment-resin solution.

Figure 4.5 showed that yellow pigment in 20% PMMA (resin) was as stable as pigment-resin added with 1% tartaric acid. The colour still maintained as original. However, pigment-resin that added with 1% citric acid faded the colour.

Figure 4.5 showed yellow pigment with bright colour in acetone. However, when mixed with resin, the colour was not so bright.

Table 4.3: Observations on different ratio of dried lotus stamen to 70% acetone. solution

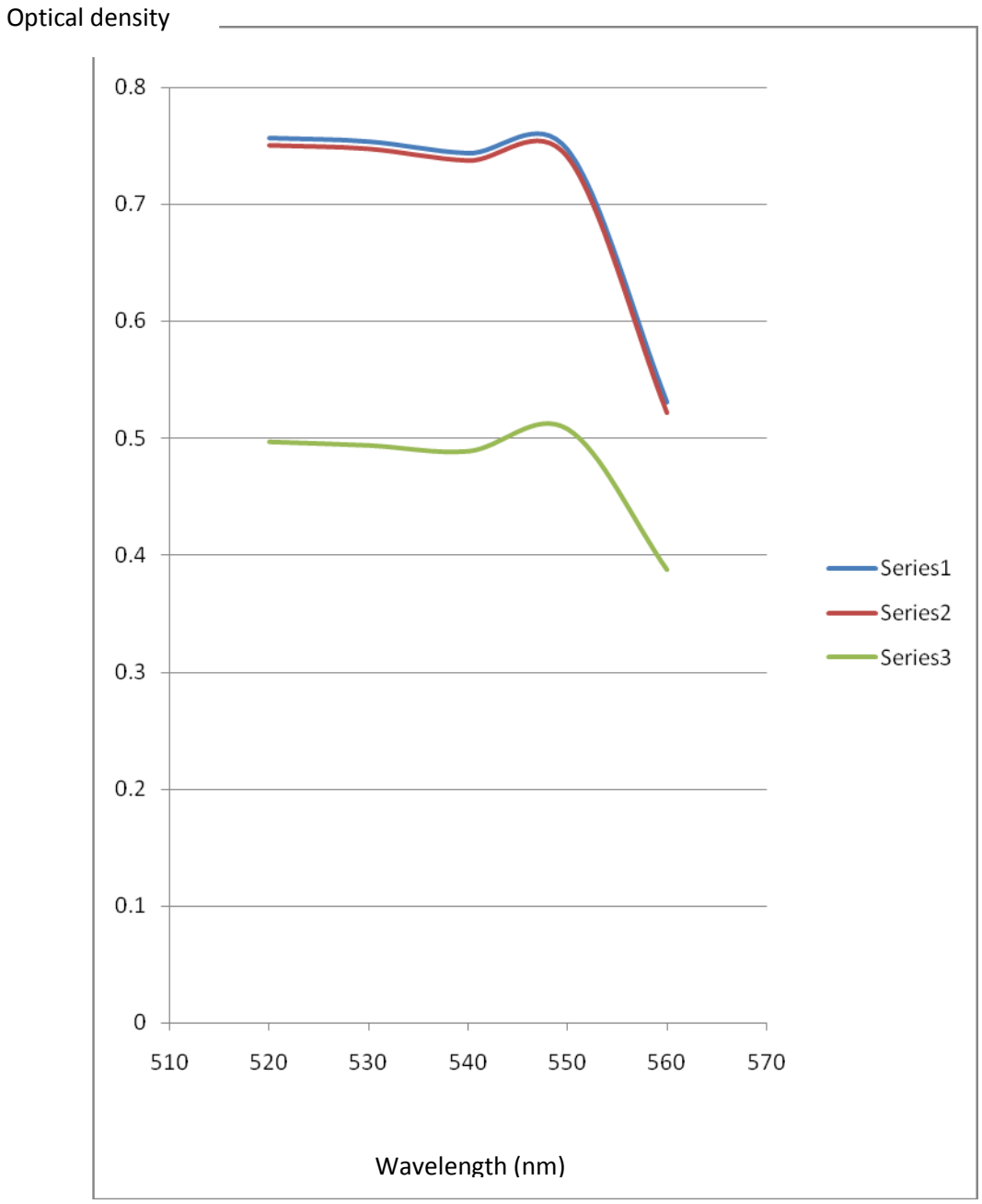
Dried Stamen (g)	70% acetone (ml)	Observations
1.0	10.0	Sample not dissolved
1.0	20.0	Bright yellow colour solution
1.0	30.0	Yellow solution

*Determination was done in three replicates

Table 4.4 : The glossiness and ratio of pigment and resin in coating process

Pigment solution (ml)	Resin solution (ml)	Glossiness,GU (mean±SE)	Observations
1.0	10.0	57.67±0.67	Smooth coating, bright red colour
1.0	20.0	67.01±0.58	Smooth coating, pink colour
1.0	30.0	74.67±0.33	Smooth coating, soft pink colour

*Determination was done in three replicates. GU-Glossiness units



Series 1: Pigment+20% PMMA(resin)
Series 2: Pigment+20%PMMA(resin)+1% tartaric acid
Series 3: Pigment+20%PMMA(resin)+1% citric acid

Figure 4.4 : Effects of 1% tartaric acid and 1% citric acid as the stabilizer for coating.

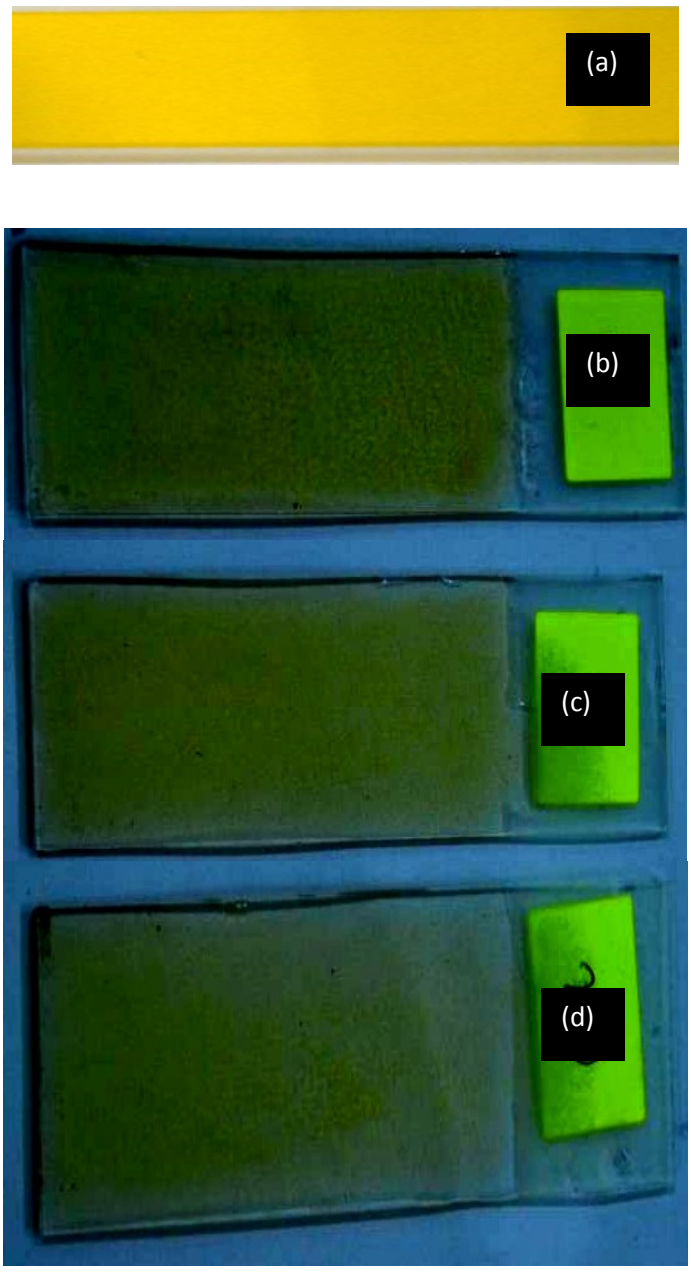


Figure 4.5 : 24 hours after coated on slide (a) Pigment in acetone solution (b) Pigment+PMMA (resin) (c) Pigment+PMMA (resin)+1% tartaric acid (d) Pigment+PMMA (resin)+1% citric acid

4.3.4 Lotus Leaves Extraction, *in vivo* and *in vitro*

Figure 4.6 showed 1g of fresh 10 cm diametres *in vivo* lotus rolled leaves (Figure 4.6 (a)) collected from Rimba Ilmu,University of Malaya and extracted in 70% (v/v) ethanol with green colour (Figure 4.6 (b)). While, 6-month-old of *in vitro* rolled leaves with 1 cm diametre (Figure 4.6 (c)) with dark green colour (Figure 4.6 (d)) in 70% (v/v) ethanol.

Figure 4.6 also showed the same character of leaves (rolled) for *in vivo* and *in vitro* with different age. Rolled leaf from *in vivo* occurred from the age of week 3-4. It growth vigorously and formed emerged and submerged leaves. Whilst, *in vitro* rolled leaf formed at the age of 4-6 months in culture. The growth was slowly at this stage.

At the end the extraction of in vivo and in *vitro* were differed with green and dark green. The effects of in vivo extraction was better compared to *in vitro* extraction, eventhough the in vitro was more matured (6 months).

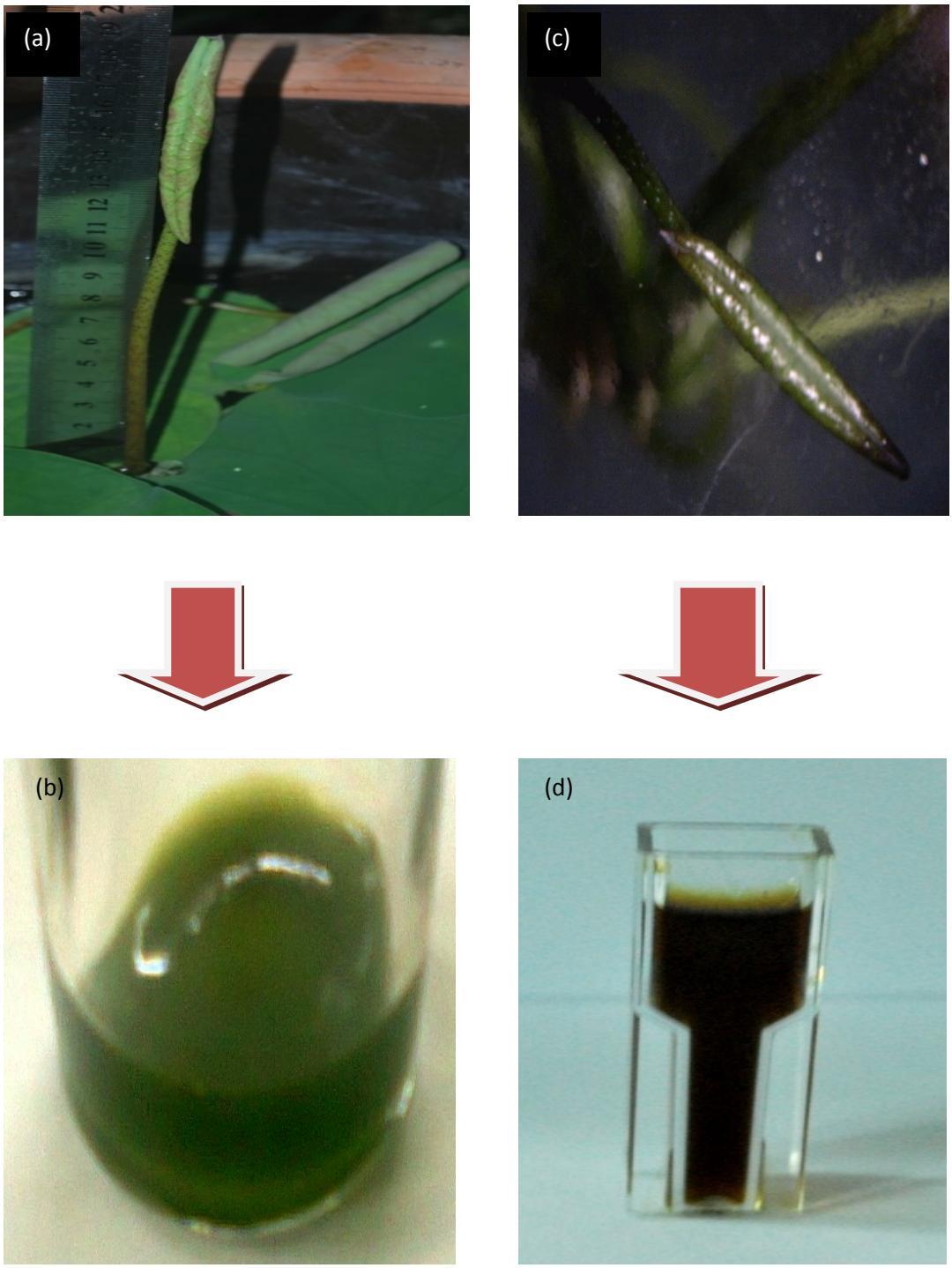


Figure 4.6 : (a) 10 cm diametres *in vivo* lotus rolled leaves obtained from Rimba Ilmu, University of Malaya and (b) green 70% (v/v) ethanol extraction, *in vivo* (c) 6-month-old of *in vitro* rolled leaves with 1cm diametre (d) dark green 70% (v/v) ethanol extraction from *in vitro* green leaves.

4.3.5 Inhibition Zone from Lotus Leaf Extracts, *in vivo* and *in vitro*

Table 4.5 showed no inhibition zone to all tested bacteria *Staphylococcus aureus, Pseudomonas aeruginosa*, *Bacillus cereus* and *Escherichia coli* for both extraction from *in vivo* and *in vitro* rolled leaves (Figure 4.8).

Table 4.6 showed the highest inhition zone was from *in vivo* sample for both on *Fusarium* sp. and *Trichoderma* sp. with mean of 9.0±0.1 mm with 7.3±02 mm diametres, respectively. Whilst, inhition zone for *in vitro* sample were 8 times lower than *in vivo* with mean of 2.0±0.4 mm on *Fusarium* sp. and 1.05±0.3 mm on *Trichoderma* sp.. Generally, *in vivo* and *in vitro* rolled leaves extracts inhibited better on *Fusarium* sp. compared to *Trichoderma* sp. (Figure 4.7).

Table 4.5 : Antimicrobial activity of hydroethanolic extracts from rolled leaves of *Nelumbo nucifera* Gaertn.,*in vivo* and *in vitro.*

Microorganisms	Inhibition zone, mm (Mean ±SE)	
	In Vivo	*In Vitro*
A. Bacteria		
Staphylococcus aureus	0	0
Pseudomonas aeruginosa	0	0
Bacillus cereus	0	0
Escherichia coli	0	0
B. Fungi		
Candida albicans	0	0
Aspergillus niger	0	0
Trichoderma sp.	**7.3±02**	1.05±0.3
Fusarium sp.	**9.0±0.1**	2.0±0.4

(Mean + SE; n=3). The bold numbers represent the best result.

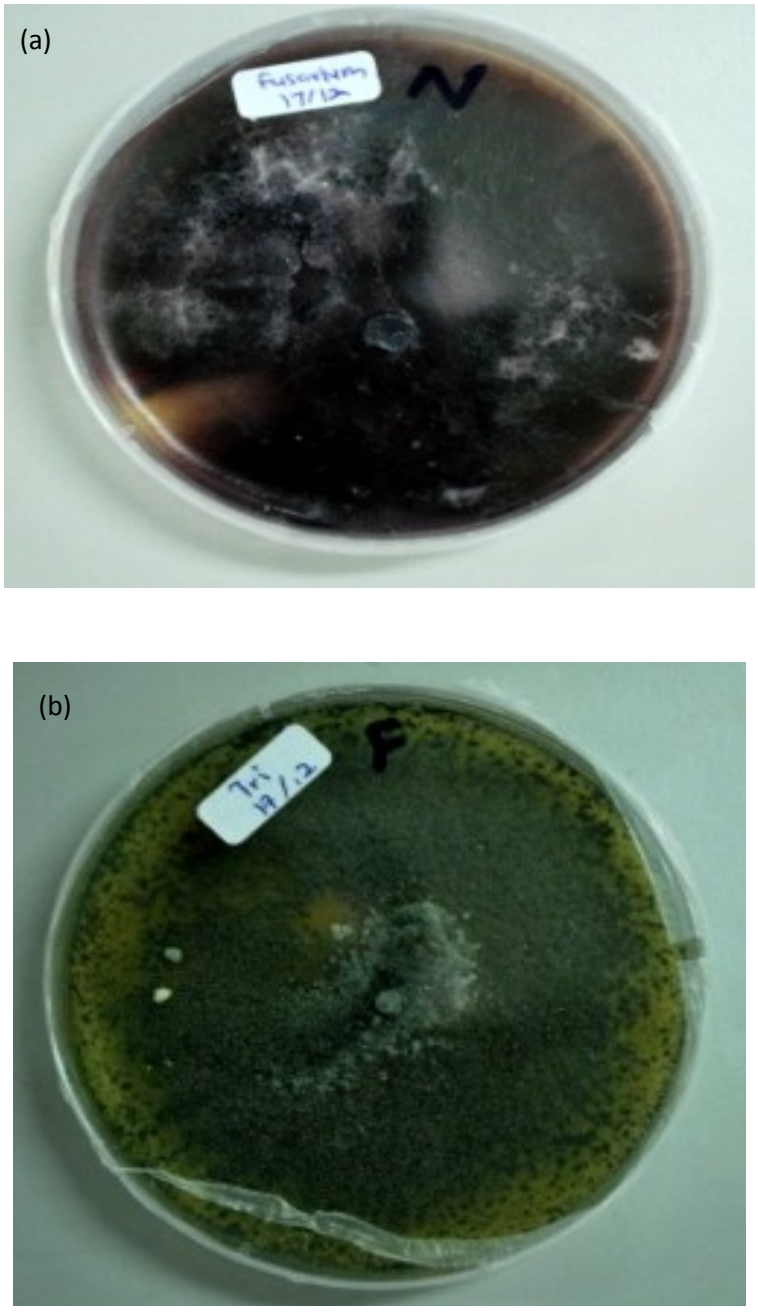


Figure 4.7 : Antifungal activity of hydroethanolic extracts of *Nelumbo nucifera* Gaertn. higher from *in vivo* rolled leaves compared to *in vitro* (a**)** *Fusarium* sp. ; mean 9.0±0.1 mm (b) *Trichoderma* sp**.;** mean 7.3±0.2 mm.

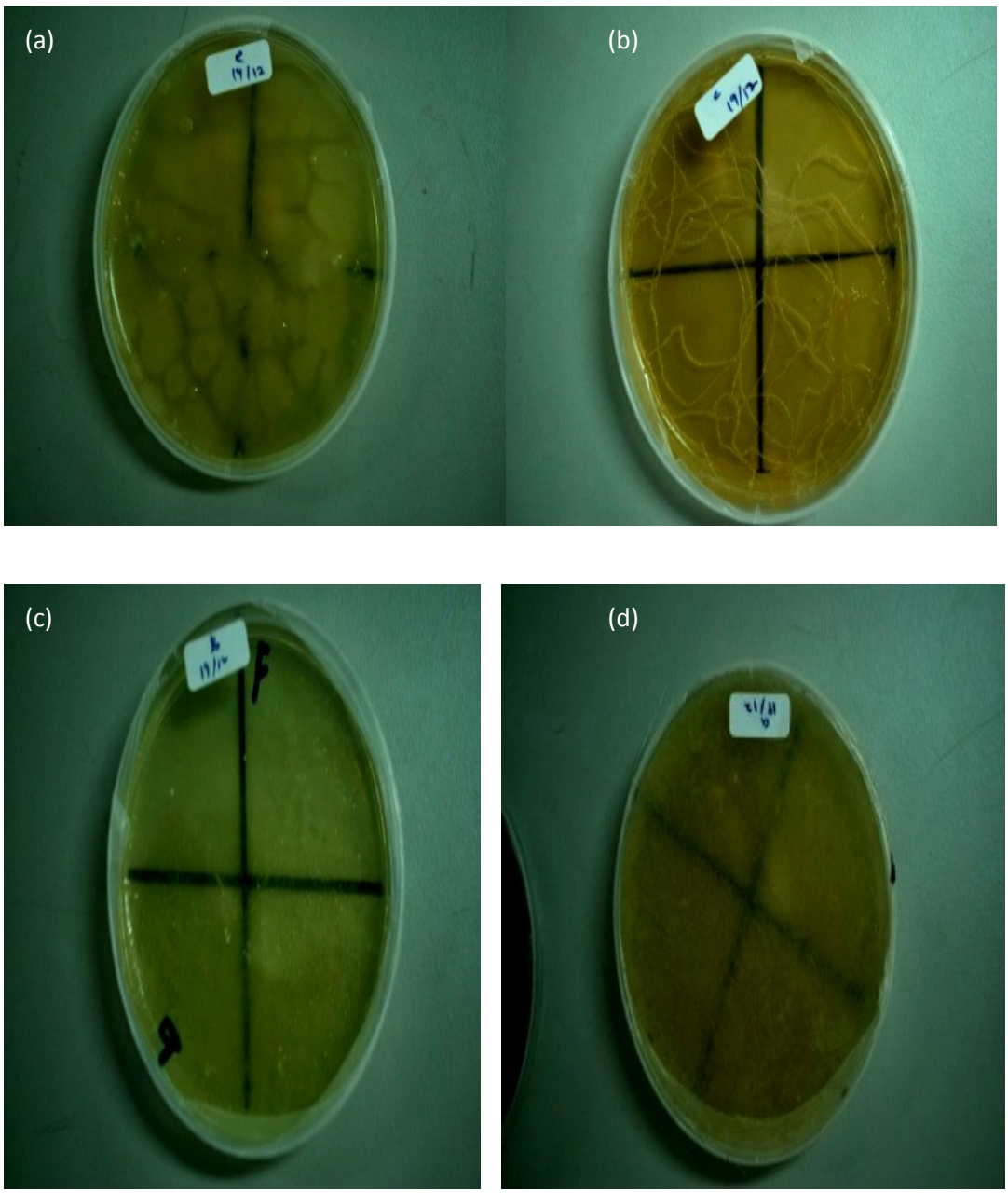


Figure 4.8 : No responses of antibacterial activity of hydroethanolic extracts from rolled leaves of *Nelumbo nucifera* Gaertn.,*in vivo* and *in vitro* (a) *Staphylococcus aureus* (b) *Pseudomonas aeruginosa* (c) *Bacillus cereus* (d) *Escherichia coli.*

4.4 SUMMARY OF RESULTS

1. Lotus flower (stamen and stigma) was found to have the total carotenoid content of 526.96 ± 0.52 µg/g DW, whereas for individual carotenoid β-carotene (460 ± 10.28 µg/g DW) was found with a relatively high concentration and neoxanthin (39.26 ± 0.82 µg/g DW) was found in lower concentrations. One unknown carotenoid was also detected.

2. Chromatography, absorption and retention time of HPLC analysis from lotus stamen contains different composition of carotenoids (6.2 minutes – neoxanthin, 26.6 minutes – unknown carotenoid, 28.1 minutes – beta-carotene).

3. At acidic condition (pH 1-6), yellow pigment changed to the darker colour, yellow-brown. While, at strong alkaline condition (pH 12-14), the colour changed to yellow-green. The optimum yellow colour was at pH 8-10.

4. The highest glossiness was in 1.0ml/30.0ml (v/v) of pigment-resin solution with mean of 74.67±0.33.

5 Yellow pigment in 20% PMMA (resin) was as stable as pigment-resin added with 1% tartaric acid, coated on glass slides.

6. The addition of 1% citric acid reduced the carotenoid color .

7. No inhibition zone was observed to all tested bacteria *Staphylococcus aureus, Pseudomonas aeruginosa*, *Bacillus cereus* and *Escherichia coli* for both extracts from *in vivo* and *in vitro* rolled leaves.

8. The highest inhibition zone was from *in vivo* sample for both on *Fusarium* sp. and *Trichoderma* sp. with mean of 9.0±0.1 mm with 7.3±02 mm diameters, respectively.

9. Inhibition zone for *in vitro* sample were 8 times lower than *in vivo* with mean of 2.0±0.4 mm on *Fusarium* sp. and 1.05±0.3 mm on *Trichoderma* sp.

CHAPTER 5

ACCLIMATIZATION OF *Nelumbo nucifera* Gaertn.

5.1 EXPERIMENTAL AIMS

Acclimatization is an adaptation process to the natural environment for various plant species which has undergone growth and development process *in vitro* (Preece and Sutter, 1991). In tissue culture system, most of the acclimatized *in vitro* plantlets showed almost similar characteristics as intact plants (Mohammed and Vidaver, 1990).

Plantlets of *Nelumbo nucifera* Gaertn. derived from tissue culture techniques were transferred to the green house for acclimatization to the natural environment. Large containers (1 metre x 1 metre) were filled in with ½ of loam or clay soil and ¼ of tap water. Plantlets covered with plastic need to be placed in culture room for 2-4 weeks before transfer to the green house to avoid stress conditions and relatively low humidity environment. Observations of nelumbo morphological features were done to compare the characteristics between intact plant and *in vitro* plants. In order to avoid pests and diseases, the greenhouse need to maintain in 25±1°C and 75% humidity.

Acclimatization in the green house could cause stress the condition for lotus plants. Flowering of lotus can be prolonged by higher temperature in the green house compared to the culture room (Li et al., 2000b). According to Yang et al. (2006), time for bud occurrence above the water level can be controlled between 10-15 days. If the techniques of advancing and delaying of lotus flowering are incorporated, three cycles of flowering are possible in a year (Wang and Zhang, 2004). Adjusting of water level based on growth stage will influence the flower quality (Chomchalow, 2004).

Medium used for plant transplanting purposed must be steriled. A chilling treatment before transplanting may improve survival rate and growth of plants. A gradual acclimatization is a critical step of transplanting and significantly affects performance of transplanted plants. After being transplanted, *in vitro* plants of *lotus* are very sensitive to fungi and bacteria attack, which spread quickly among the plantlets if a nonsterile environment exists. Therefore, individual acclimatization in separate pot or multi-plots was suggested to reduce disease development (Albers and Kunneman, 1992). Low temperature, high humility and long day length also may benefit transplanting

All 8-month-old *in vitro* plantlets regenerated in 0.5 mg/l BAP obtained from earlier experiments were transferred to the green house for the acclimatization process. Eight different type of soil were tested for optimal growth characteristics observations.

The objective of this chapter was to obtain the most suitable technique of acclimatization for lotus plantlets regenerated from tissue culture system (*in vitro*).
The major objectives of this study were:

1. To investigate the lotus growth in containers influenced by type of soil, soil level, water level, pH, water temperature and nutrients or fertilizers in contributing for nursery or plantation industry.

2. To establish the plantlets from *in vitro* system to the green house or natural environment for the best adaptation or acclimatization.

3. To prepare plant stocks in containers or pots with easy maintenance for nursery industry in Malaysia.

5.2 MATERIALS AND METHODS

5.2.1 Source of *In Vitro* Regeneration

Eight-month-old *in vitro* plantlets on MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA were washed under tap water. For the first 2-3 weeks, plantlets were transferred to the soil and covered with plastic in the culture room. After that, plantlets exposed to the natural environment in the green house.

5.2.2 Transplanting Plantlets to Containers

Nelumbo nucifera is an aquatic plants with horizontal slender, vegetative stolon and thickened storage tuber. It consist of submerged and emerged petioles and two different forms of leaf (lamina), floating and emergent leaf (heterophylly). The leaves were float on the water surface at the immature stage and later were raised higher (emerged). The unique adaptation to the deeper water level is through the elongation of the petiols from underwater to the water surface (merged and submerged). In the present study, plantlets were transferred to aquarium and water container for the growth observation. The survival rates were influenced by soil types, pH and sunllight exposure.

5.2.2.1 Determination of Sterile Soil Type and pH on Survival Rates of Green Shoots and Yellow Shoots

Eight types of soil were autoclaved at 121°C for 21 minutes at 104 kPa (15Psi^2). Cultures were maintained in culture room for 21 days before being transferred to the green house. Thirty replicates for each experiment.

A. Original soils collected from different research sites:

1. Clay Soil – cream in colour, sticky and compact in texture
2. Mud Soil – black in colour, bad smell, sticky and compact texture
3. Burn Soil – black incolour, light texture
4. Top Soil – brown in colour, medium light texture
5. Sand – white in colour, porous

B. Treated soils bought from Fern Garden Nursery, Sungai Buluh, Selangor, Malaysia

1. Black Clay Loam (heavy loam) – black in colour, sticky and compact texture
2. Black Soil – light and porous texture
3. Red Soil– sticky and compact texture

5.2.2.2 Effects of Sunlight Exposure on Stem Height and Leaf Width

In this experiment, 4 month-old and 8 month-old plantlets were transferred to heavy black clay loam soil (with 2:1 ratio solid:liquid) and placed in the shady and full sunlight area in the green house (30±1°C). Measurement on the growth of stem height and leaf width were done. Three replicates for each treatment.

5.2.2.3 Growth Development of Plantlets

In this experiment, observations on lotus growth in fresh water aquarium (clear medium) and 50 cm diameters of clay containers were done. The development of lotus parts (roots, petioles, stems and leaves)were studied.

5.2.3 Data Analysis

All experiments were conducted using a completely randomized design. Data collected were statistical analyzed using Duncan's Multiple Range Test (DMRT). Mean with different letters in the same column differ significantly at p=0.05. Thirty replicates for each treatment.

5.3 RESULTS

5.3.1 Determination of Soil Type and pH on Survival Rates of Green and Yellow Shoots

Table 5.1 showed eight types of soil which were collected from the original habitats of samples at different pH and survival rates of the plantlets. The pH ranged from 3-7. The most acidic soils were collected from Tasik Chini with mean pH of 3.97±0.02 with the lowest survival rate, 31.34±0.77% for green seeds and 11.11±0.51% for yellow seeds .Whilst, black mud, white sand and black burn soil were with mean pH of 4.26±0.12, 4.57±0.13 and 4.89±0.11, respectively. The less acidic soil were red soil or laterite with mean pH of 6.38±0.27, followed by black clay loam with mean pH of 6.03±0.29, black topsoil with mean pH of 5.14±0.12 and black soil with mean pH of 5.08±0.18. The highest survival rate was in black clay loam for both plantlets from green and yellow seeds with 83.01±0.23% (Figure 5.1) and 69.22±0.43% (Figure 5.2), respectively. Soil samples collected from Tasik Chini with mean pH of 3.97±0.02, while the optimum survival for acclimatization of plantlets was on black clay loam (mean pH 6.03±0.29) that normally commercialized at all the nurseries in Malaysia (Figure 5.3).

Table 5.1 : Effects of soil pH on survival of acclimatized plantlets from green seeds and yellow seeds.

Type of Soils	Original Habitat	pH (mean±SE)	Survival Rates (%±SE)	
			Green Seeds	Yellow Seeds
Cream Clay Soil	Tasik Chini, Chini, Pahang	3.97±0.02	31.34±0.77f	11.11±0.51de
Black Mud Soil	Batu 10, Segamat, Johor	4.26±0.12	40.23±0.31e	13.23±0.53d
Black Burn Soil	AMJ highway, Muar, Johor	4.89±0.11	64.22±0.15c	36.52±0.72c
Black Topsoil	Rimba Ilmu, UM, Kuala Lumpur	5.14±0.12	72.11±0.13b	47.04±0.25b
White Sand	UTP, Tronoh, Perak	4.57±0.13	56.37±0.44d	16.57±0.11d
Black Clay Loam	Fern Garden Nursery, Sungai Buluh, Selangor	6.03±0.29	**83.01±0.23a**	**69.22±0.43a**
Black Soil	Fern Garden Nursery, Sungai Buluh, Selangor	5.08±0.18	70.21±0.55b	40.03±0.37c
Red Soil	Fern Garden Nursery, Sungai Buluh, Selangor	6.38±0.27	29.36±0.25f	10.01±0.15de

mean±SE, n=30. The bold numbers represent the best result.

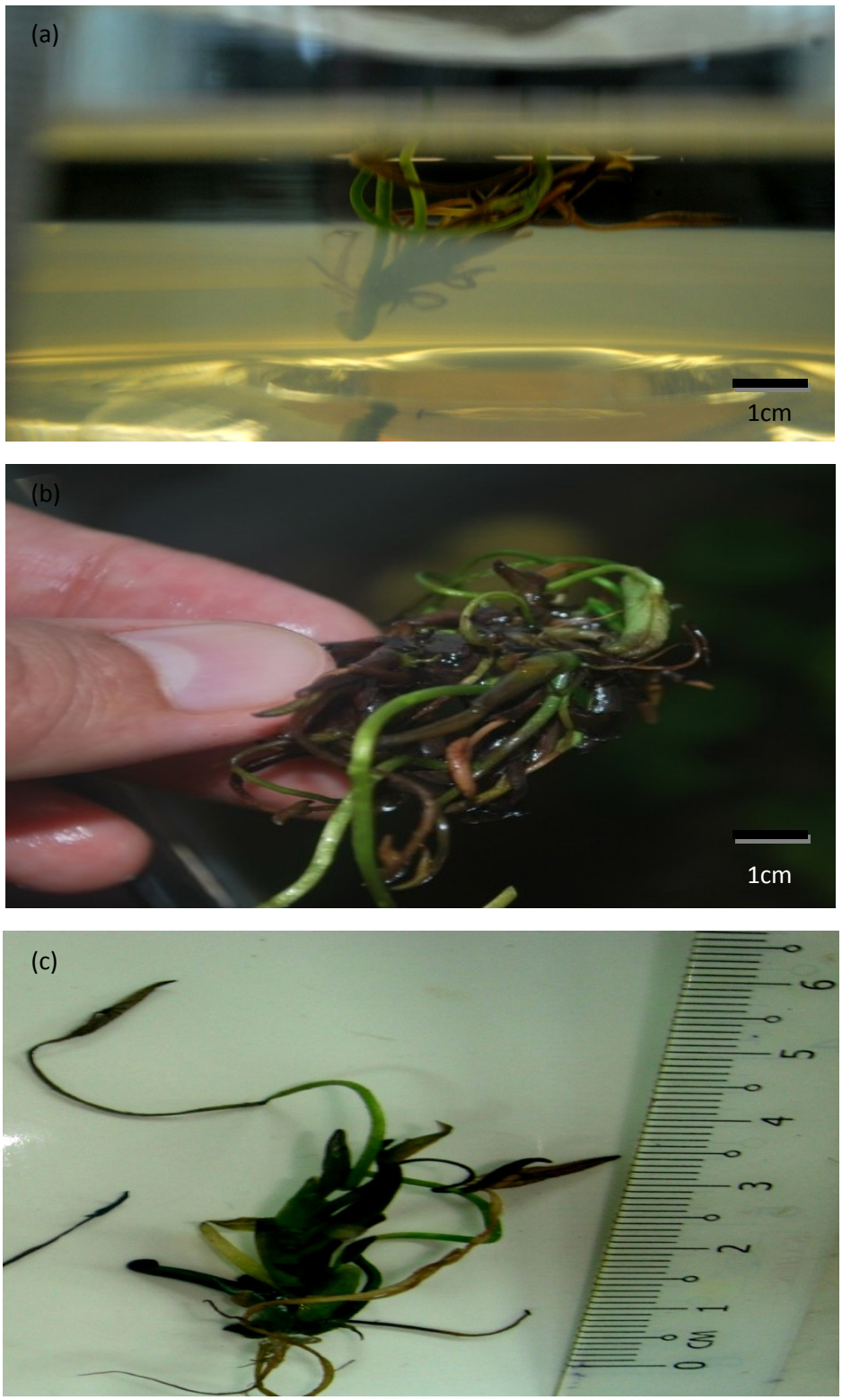


Figure 5.1 : 8-month-old plantlets in double layered media (solid:MS+0.5mg/l BAP+1.5mg/l NAA, liquid:MS basal media) from yellow seed explants. (a) cloudy solid agarose MS media and clear liquid MS media (b) clumps of multiple shoots and roots; washed with tap water (c) layered multiple shoots (new characteristic; differ from mother plants).

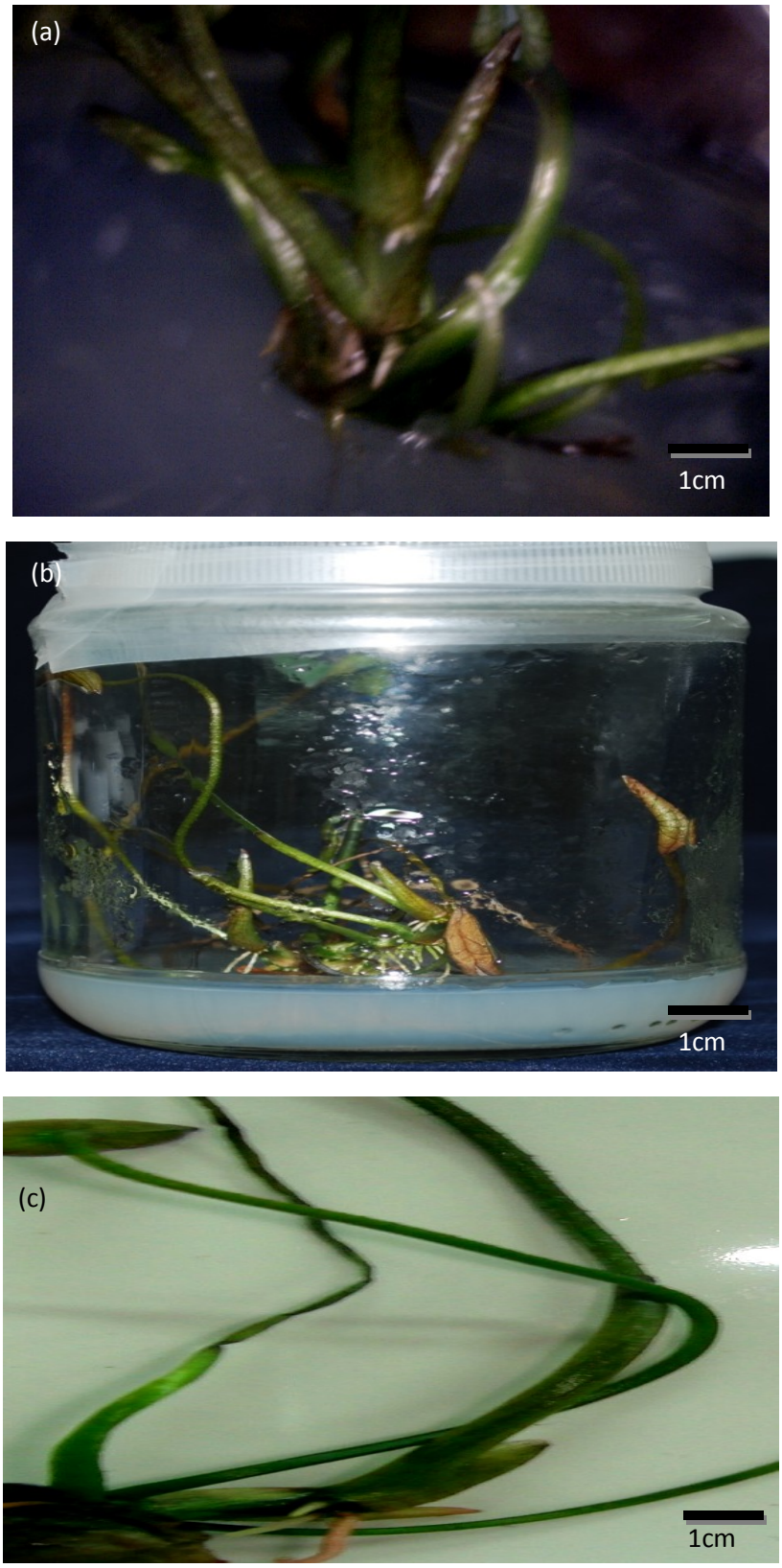


Figure 5.2 : 8-month-old plantlets cultured on solid MS media supplemented with 1.5mg/l BAP 0.5mg/l NAA, from green seeds (a) multiple shoots (b) true-to-type creeper plantlets with horizontal stem and rolled leaves (c) internode; runner with new roots.

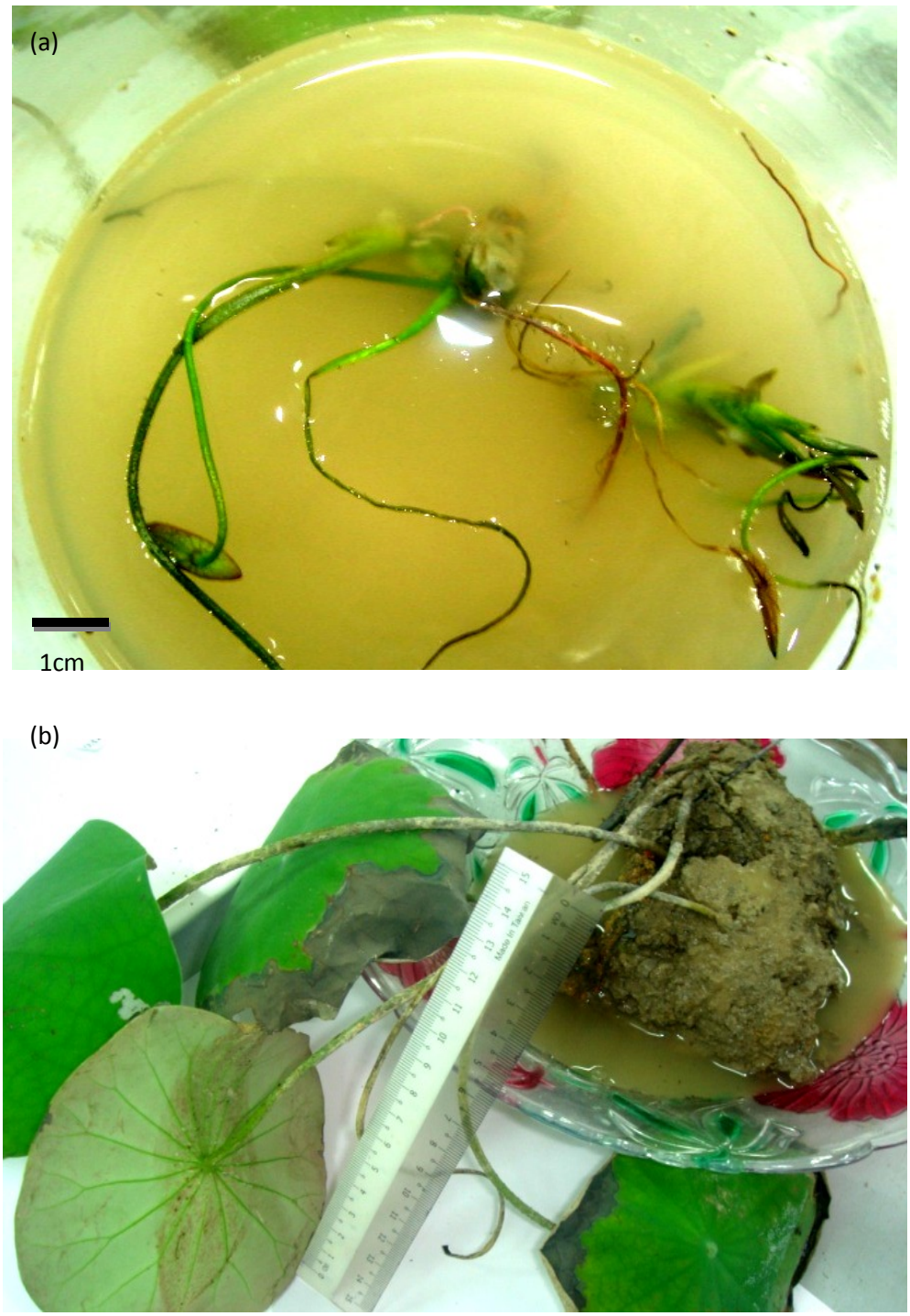


Figure 5.3 : Acclimatized plantlets in clay loam with ratio 2:1, (solid to liquid) for adaptation to natural environment (a) 14th days uncovered plantlets in culture room (25±1°C) for initial adaptation. Plantlets were transferred to green house after 21 days (b) 6-month-old plantlet in green house (with 4 unrolled leaves and fibrous roots).

5.3.2 Effects of Sunlight Exposure on Stem Height

Figure 5.4 and Figure 5.5 showed the comparison of acclimatized lotus in stem height under full sunlight and shade area at 30±1°C, in black clay loam soil with 2:1, ratio solid to liquid. After 6 months being acclimatized, the highest height was for acclimatized under full sunlight exposure with mean of 147.03±0.45cm with eight unrolled leaves and four floating leaves. While, mean of 17.66±1.71cm in shady exposure with two floating leaves. The lowest height with mean of 24.78±0.19 cm under full sunlight exposure and mean of 4.37±0.24 cm in shady exposure.

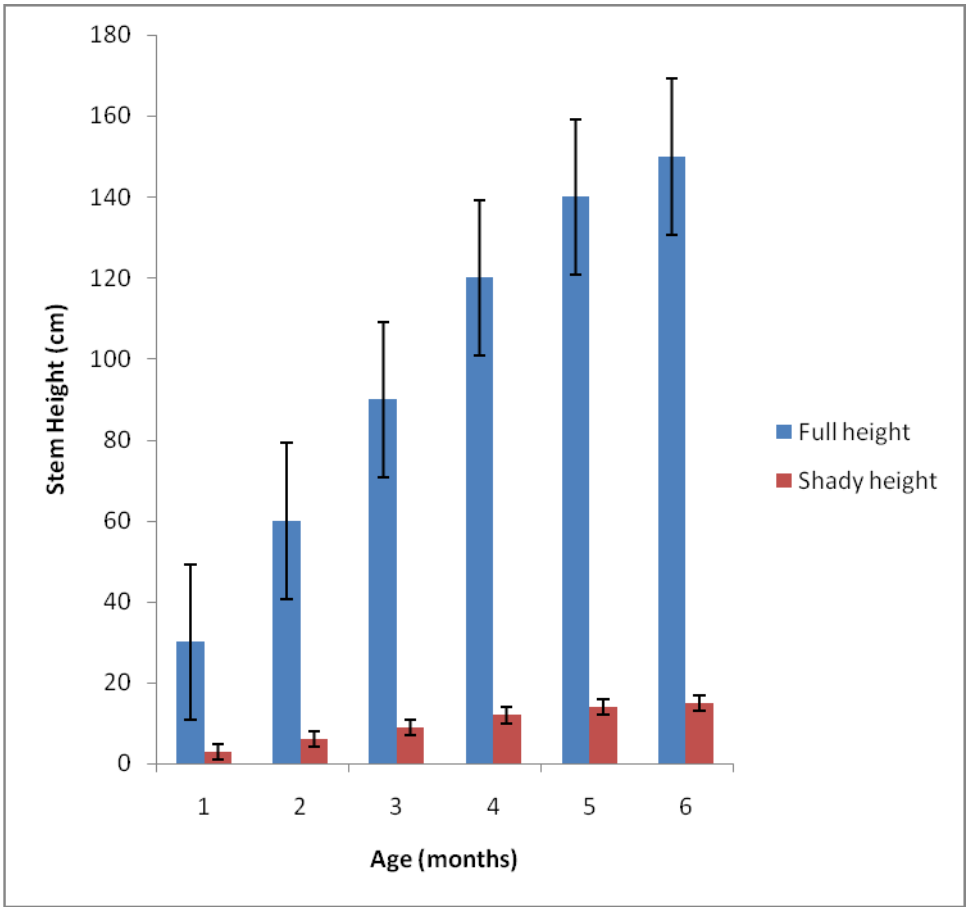


Figure 5.4 : Comparison of acclimatized lotus in stem height under full sunlight and in the shade at 30±1°C.

Figure 5.5 : Effect of full sunlight on growth of lotus plants in clay loam with ratio 2:1 (solid to liquid) (a) after 8 months being acclimatized under full sunlight with 12 fresh leaves and 4 rotten leaves. b) after 8 month being acclimatized under shady greenhouse with 2 leaves only.

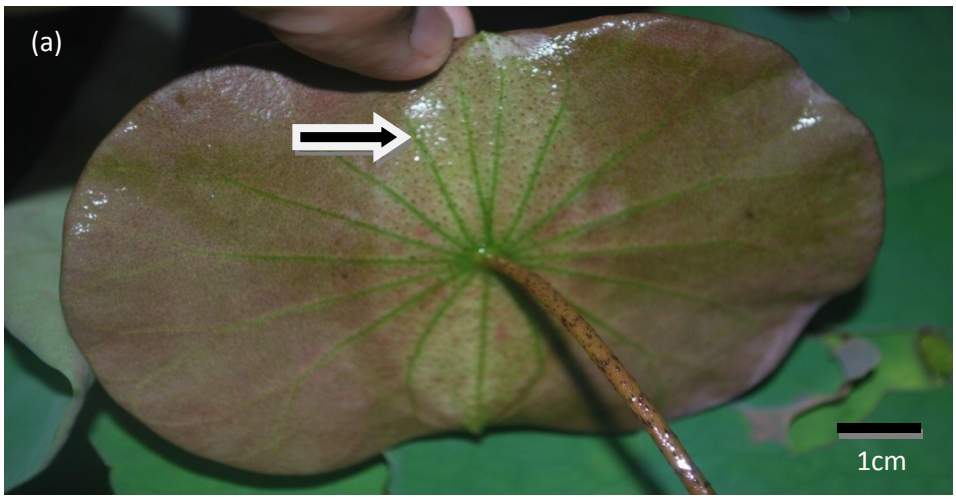
5.3.3 Transplanting Plantlets to Containers

Table 5.2 showed development of plantlets transferred to containers (fresh water aquarium and water container. For the first 8 months being acclimatized, stems and leaves grew vigorously with red spotted leaves and hollow stems (Figure 5.6). At early stage, similar observations were made when transferred to aquarium and container (Figure 5.7). However, when the leaves emerged from water surface, the leaves became only green in colour (Figure 5.8).

Table 5.2 : Growth of stems and leaves of different ages.

Age (month)	No. of stem-leaf (mean±SE)	Leaves width (cm) (mean±SE)	Stem (cm) (mean±SE)	
			Height	Width
2	4.01±0.31	4.12±0.35	30.32±0.65	0.30±0.01
4	8.52±0.11	8.41±0.67	60.23±0.57	0.61±0.20
6	12.09±0.23	12.50±1.23	90.14±1.71	0.92±0.07
8	16.30±0.34	16.27±0.87	120.65±0.62	1.23±0.81
10	20.11±0.13	20.73±0.36	140.41±0.38	1.31±0.92
12	24.42±0.17	30.12±0.08	150.17±0.04	1.52±0.77

Mean ± Standard error (SE), n=30.



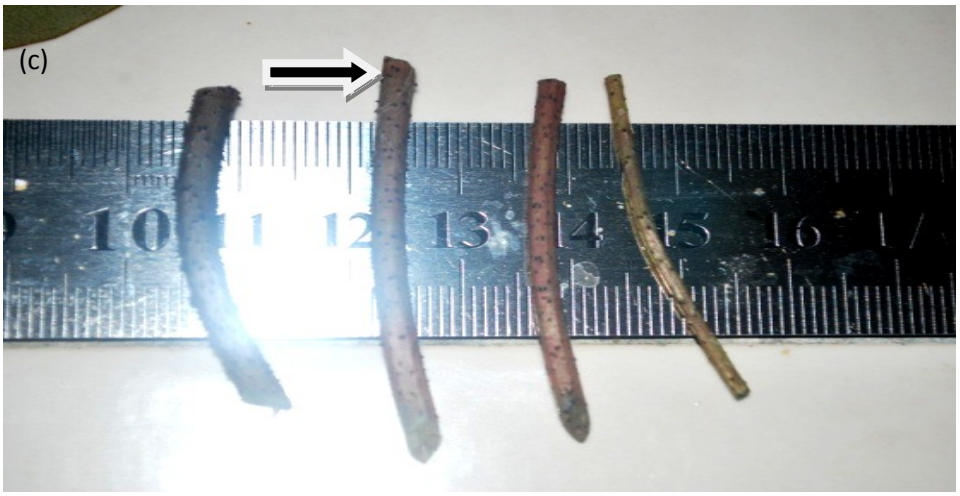


Figure 5.6 : First 4 leaves of 8-month-old acclimatized plantlets, emerged from water surface (a) adaxial of leaves: red spotted (b) abaxial of leaves: waxy green (c) stem: hollow and prickles (tiny torns).

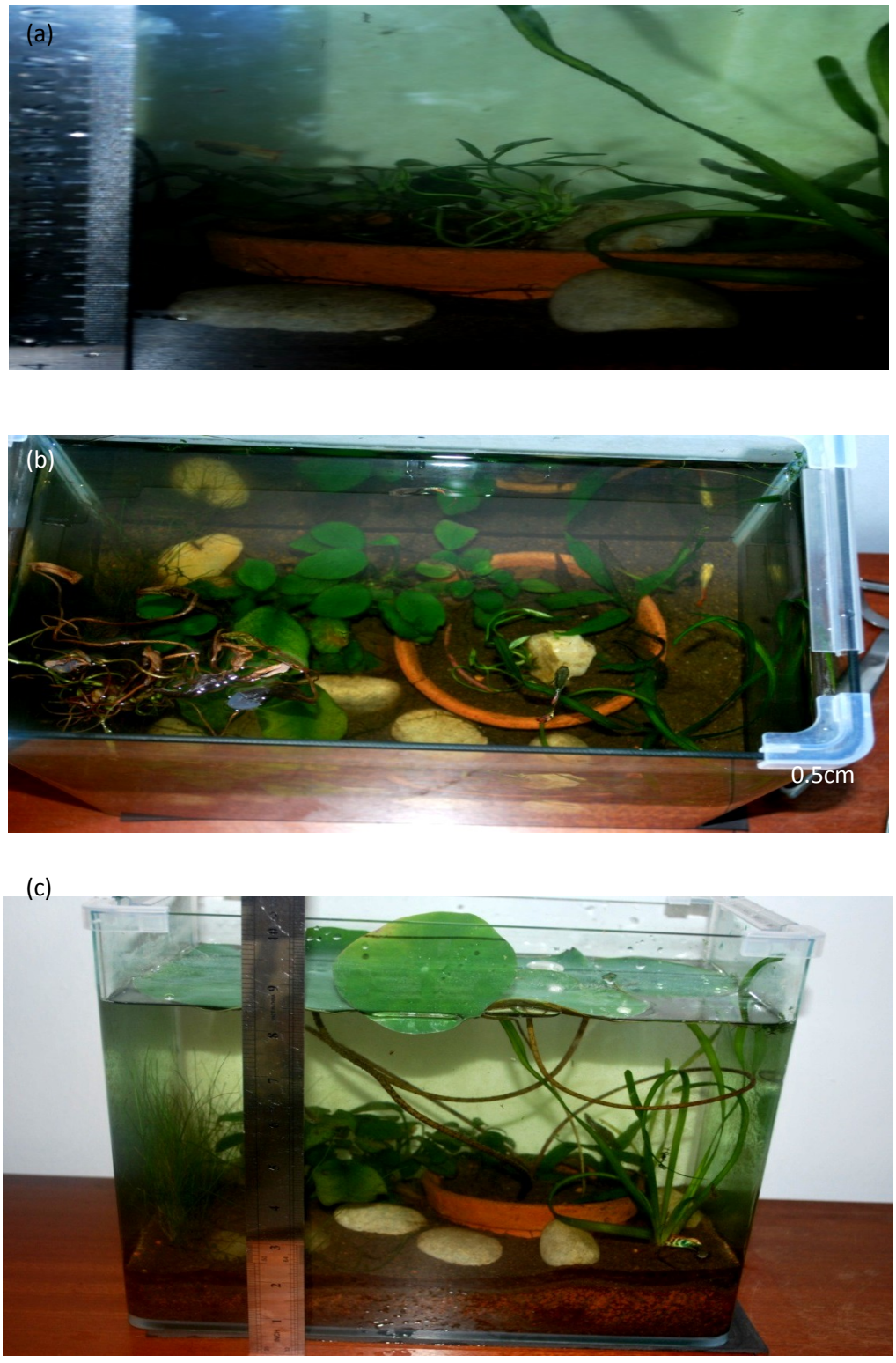


Figure 5.7 : Acclimatization in fresh water aquarium mixed with black clay loam soil and associate with guppy fish. (a) 8-month-old plantlets (b) shoots elongation (c) plantlets after 8 months acclimatization.

Figure 5.8: 8 months of plantlets acclimatization in containers a) green floating leaves b) first 3 leaves (emerged), 1 rolled leaf and 1 petiole.

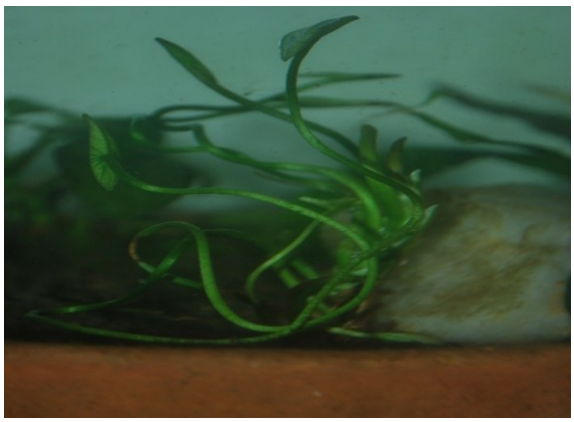
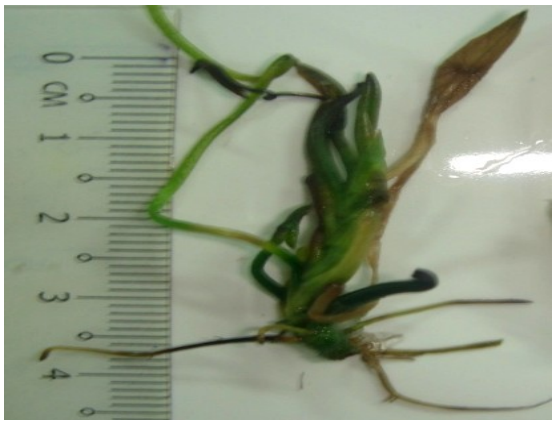
5.3.4 Growth Development of Lotus Plantlets

Table 5.3 indicates the observations on lotus characteristics after 8 months acclimatization under full sunlight exposure. All the acclimatized plantlets showed the same characteristics and adaptations as mother plants (Figure 5.9) within 8 months acclimatization under full sunlight in clay loam soil (mean pH 6.03±0.29). Even though at the beginning were transferred with layered multiple shoots. However, it was a temporary new character.

Table 5.3 : Observations on lotus characteristics after 8 months acclimatization under full sunlight exposure.

Age	Observation on Lotus Parts, Characteristics and Adaptation
8-month-old, *in vitro* plantlets (Figure 5.9 (a))	**1. *In vitro* plantlets** *plantlets with primary and secondary roots, 8-20 rolled leaves and 1-3 unrolled leaf *previously cultured in double layered MS media (solid ;MS+1.5mg/l BAP+0.5mg/l NAA, liquid; MS basal) and transferred to clay loam soil in culture room (25±1°C, 16 hrs light with distance 2.0-2.5 cm for 21 days, uncovered)
2-month-old, after acclimatized (Figure 5.9 (b))	**2. Runner** Condition: runner creeps on the soil surface, under water (submerged) Characteristics: * runner with pink in colour and sharp at the end Adaptation: *grow as horizontally stolon. Finally, developed into rhizome
2 1/2 month-old ((Figure 5.9 (c))	**3. Petiole** Condition: Submerged (under water) and merged (on water surface) Characteristics: *to keep laminae (leaf)on the water surface. *Green petiole will elongated to the water surface depending on water depth. *emerged petiole unrolled and performed as floating leaf
3 month-old ((Figure 5.9 (d))	**Stem** Condition: Submerged and emerged Characteristics: *Perennial, milky latex present, dimorphic: slender, hollow and black small torn along it. Adaptation: *very light and easy to move with water support for petiole and leaf elongation. Low diffusion of oxygen in water
4-month-old ((Figure 5.9 (e))	**4. Leaf** Condition : Submerged and emerged Characteristics: *Oval Green waxy leaf (membraneous). net vein (numerous small spines). Two types of leaf: ii) leaf on water surface: bright red spot at the back of the leaf ii) emerged leaf: *Adaptation: lotus can transfer oxygen-rich air from emergent or floating leaves to young submerged leaves via stolon and tubers
8-month-old ((Figure 5.9 (f))	**5.Roots** Condition : grown underground Characteristics : *Fibrous roots which extended as horizontal stolons or rhizomes, or thick storage rhizomes;. Adaptation: *Roots can grow both directly in the water and at edge of lake (soil)

(a) Plantlet

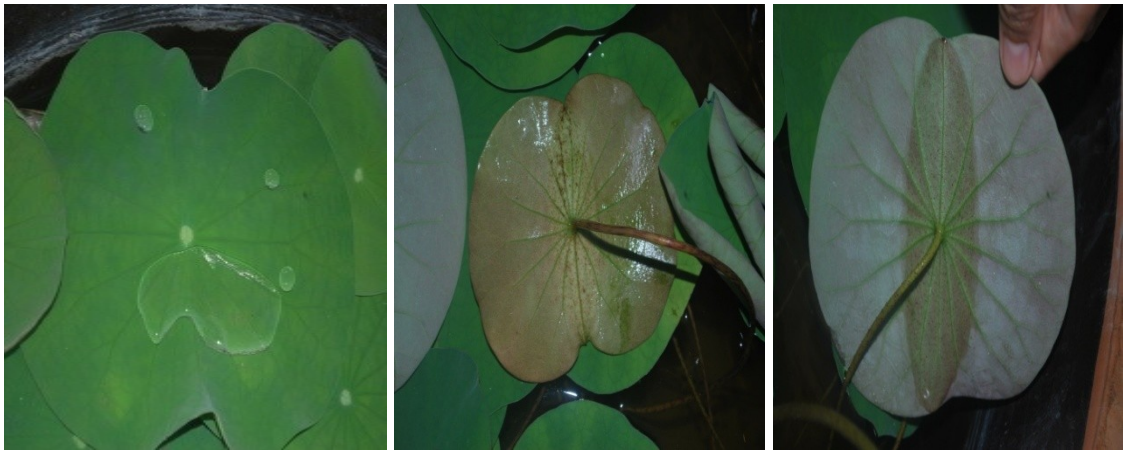
(b) Runner


(c) Petiole



(c) stem

(d) Leaf



(f) Roots

Figure 5.9 : Lotus characteristics and adaptations within 8 months acclimatization under full sunlight in clay loam soil (mean pH 6.03±0.29).

5.3.4 SUMMARY OF RESULTS

1. Soil samples were collected from Tasik Chini with mean pH of 3.97±0.02, while the optimum survival for acclimatization plantlets was on black clay loam (mean pH of 6.03±0.29) that commercialized at all the nurseries in Malaysia.
2. The most acidic soil was collected from Tasik Chini with mean pH of 3.97±0.02 with the lowest survival rate, 31.34±0.77% for green seed and 11.11±0.51% for yellow seeds.
3. The highest survival rate of plantlets was in black clay loam for green and yellow seeds with 83.01±0.23% and 69.22±0.43%, respectively.
4. The highest plantlet's height was observed after being acclimatized under full sunlight exposure with 10 times compared to under the shade.
5. Acclimatized plantlets showed the same character as mother plants. Even though at the beginning were transferred with layered multiple shoots. However, it was a temporary new character.

CHAPTER 6

DISCUSSION

The present research deals with *in vitro* germination, regeneration from tissue culture system and pigment detection through HPLC analysis. The wild seeds of *Nelumbo nucifera* Gaertn. (pink Asian lotus), were collected from Tasik Chini, Pahang, Peninsular Malaysia.

Table 2.1 (Chapter 2) showed seeds were treated with eight various techniques in breaking the seeds dormancy; soak 24 hours in distilled water, soak in warm water for 24 hours, soak 2 months in distilled water scarified with medium-grade sandpaper, moist-prechilling in 4±1°C for 2 months, soak for 2 minutes in merchuric chloride, soak 2 min in 99% (v/v) chlorox and soak 2 min in 70% (v/v) ethanol. Overall, disinfection and contamination for green seeds was lower than black seeds. The presence of numerous musilage secreting trichomes in *Nelumbos* species cause difficulties in surface sterilization. Hard surface from matured seeds (black), also have been attributed to the presence of large bacterial and fungal epiphytic populations, muscilogenous coatings, endophytic organismsin highly lacunate tissues and lack of acutinized epidermis and causes contamination to the cultures (Godwill and Willis, 1964).

Technique of moist-prechilling with soaked in 4±1°C for 2 months, followed by exposure to room temperature (30±1°C) were response slightly lower from the scarified seeds. Dormancy and growth of *Nelumbo lutea* (American lotus) was investigated by Meyer (1930) which showed that higher temperature (20-30°C) were found to greatly accelerate growth, while growth was limited below 15°C. Black seeds (Figure 2.3) were germinated with 41.16±0.17% *in vivo* and 43.15±0.13% *in vitro*. Followed by green seeds with 33.67±0.23% *in vivo* and 34.63±0.41% *in vitro*. Yellow seeds were only germinated *in vitro* with the lowest rate of 38.52±0.31%. Whilst, ,technique of soaking in 2 months distilled water were only response to black seeds with swollen seeds at germination rate 44.01±0.27% *in vivo* and 66.01±0.12% *in vitro*. Huang et al. (2003) found that lotus seeds had an unusually strong heat resistance for germination.

Soaking for 2 minutes in 0.1% (w/v) mercuric chloride, 99% (v/v) chlorox and 70% (v/v) ethanol only response to green seeds with 26.31±0.23% *in vivo* and 33.33±0.17% *in vitro*, 23.33±0.21% *in vivo* and 25.06±0.13% *in vitro*, and 21.31±0.23% *in vivo* and 22.33±0.23% *in vitro*, respectively. According to Masuda et al. (2006), high temperature and long day length accelerated vegetable growth and short day length, was the main environmental factor leading to induction of dormancy in lotus plants.

Table 2.1 (chapter 2) showed both seeds of black and green seeds gave response with highest germination rate *in vitro* condition compared to natural habitat. The black and green seeds, both gave the highest response to technique of scarified with medium-sand paper. Germination from black seeds with rate of 67.01±0.28% *in vivo* and 78.35±0.61% *in vitro*. Whilst, germination from green seeds were higher with

75.12±0.16% *in vivo* and 100.00% *in vitro*. No germination observed from whole seeds in soaking 24 hours in distilled water (control). However, in this research solid Murashige and Skoog (1962) media was optimum for the seeds germination compared to liquid MS media. Contrary reported by Francko (1986a) that 98% germination of lotus seeds in sterile liquid culture, according to the natural habitat (fresh water plant).

Table 2.2 showed the response of three types of lotus seeds on solid basal media. Matured black seed pods (Figure 2.4 (a)), immature green pod (Figure 2.4 (b)) and young yellow pods (Figure 2.4 (c)) contains 8-40 seeds depends on the size of growth. The whole seeds without any scarified (Figure 2.4 (d)) were act as control (no response). The seeds of lotus can maintain viability for decades with life span between 100-1300 years have been reported (Ohga, 1923; Priestley and Posthumus, 1982; Shen-Miller et al., 1995; Li et al., 2000a).

The seed with inner layer or coatless (Figure 2.4 (g)) changed the white layer to brown and finally black because of the phenolic compound (oxidized). The highest shoot length was for seeds without cotyledon (Figure 2.4 (f)) with 28.00±0.55mm for black seeds, 26.00±0.16 mm for green seeds and and 14.43±0.09 mm for yellow seeds. The lowest shoot length was in seeds with one cotyledon (Figure 2.4 (h)) with 15.33±0.23 mm for black seeds and 14.00±0.15 mm. For yellow seeds, no shoot elongation since the seeds turned to brown and died. No formation of roots for all seeds in this treatment. White cotyledon changed to green on solid MS media for both explants, green seeds (Figure 2.4 (h)) and yellow (Figure 2.4 (i)), after 1 week.

Germinating studies of lotus seeds *in vitro* useful for manipulation of the physiological and morphological characteristics. According to Pristley (1986), the

average seed longevity under laboratory conditions normally between 2-10 years. The mechanism of longevity from lotus seeds remains unknown but possible explanations were given by Li et al. (2000c) who reported that high level of superoxide dismutase (SOD) activities in seeds might benefit the long life span of lotus seeds. The high stability of SOD in radicles observed under high temperature is possibly related to the longevity of lotus seeds. Biological characteristics of the centuries' seeds, germination, growth and development of the seedlings, and morphology of offspring have been studied by Shen-Miller et al. (1995, 2002), Li et al. (2000c), and Nagashima (2001).

According to Meyer (1930), dormancy and growth of *Nelumbo* was largely influenced by pH, temperature and soil types. Table 2.3 showed the effects of different pH on germination of seeds on solid MS basal media of three types of lotus seeds according to the age and maturity. Both black and green seeds responded to all of the media with pH 4.5, 5.5, 6.5, 7.5 and 8.5. From Table 2.3 and Figure 2.5, the highest shoot length was in media with pH 5.5 for black seed (64.03±0.02 mm), green seeds (32.55±0 mm) and yellow seeds (11.11±0.03 mm). These were followed by media with pH 4.5 with 50.01±0.23 mm for black seeds and 26.88±0.51 mm for green seeds. In pH 8.5, the results gave the lowest with 22.31±0.02 mm and 11.33±0.31 mm. However, young yellow seeds turned black and died. Different plant species have different adaptations to pH level in the growth substrates or soil. Some plants require acidity condition, while others need more alkaline environment for growth. Lotus can tolerate pH 4.5 to 9.0 (Meyer, 1930). The pH-dependent of plants grown in acidic and alkaline liquid media were statistically similar (Francko, 1986b).

The germination rates of *Nelumbo nucifera* were dependent on the type of seeds (age of seeds). According to Wu (2003), browning was caused by oxidization of the total phenol by catalysis of polyphenol oxidase (PPO) while oxygen existed. The total phenolic content decreased and PPO was active during browning. Table 2.4 showed the response of primary root induction on solid MS basal media after 12 weeks in culture. All type of seeds responsive to the media with 1 mm root elongation per day. The maximum root length for both black and green seeds were 14 mm on the 14^{th} day with 3 green shoots and 6 white primary roots. While, maximum root length for yellow seeds were 17 mm on the 18^{th} day with 3 green shoots and 6 white primary roots. When the roots stopped elongated, dark green shoots became stunted. Figure 2.6 showed the standard root growth.with root formation started after 10-24 weeks in culture depending on the age of the seeds.

Besides germinating the whole seeds of lotus (conventional method), synthetic seeds technology through encapsulation matrix had been study on lotus petioles from immatured seeds (green) to ensure the viability of lotus propagules. Propagules could be stored in the low temperature (4 ±1°C), for up to 6 months. After 3 months of storage, there were no decayed propagules, and 94% of total propagules maintained their viability after planting. Water retention and shelf-life of micro shoots were similar among all treatments during storage.

Research on synthetic seeds can be useful for the large scale propagation of superior hybrids. Synthetic or artificial seeds have many other significant applications. Synthetic seeds derived from microshoots or somatic embryos can be created from very small pieces of plant tissue, to serve as a supplement to the already existing propagules,

to prevent extinction of endangered species, to increase productivity and to propagate plants with low seed viability and expensive imported seeds. Synthetic seeds can also be used for cryopreservation of elite genotypes. Synthetic seeds can also be used as artificial propagules for plants which reproduce asexually. Synthetic seeds consist of viable plant parts and artificial endosperms which can be germinated when necessary. The synthetic seeds can be stored under low temperature (4°C) and germinated throughout the year and independent of seasons. Synthetic seeds can be produced for elite genotypes of this species to ensure the uniformity and clone nature of the offsprings cultivated in the field. The most outstanding advantage is the capability of small pieces of vegetative tissues to produce hundreds or more artificial propagules through plant tissue culture technology.

Rao and Purohit (2006) reported that the encapsulated shoot tips can be handled like a seed and could be useful in minimizing the cost of production as 1 ml of medium is sufficient for encapsulation of a single shoot tip compared to 15-20 ml for conversion of shoot tips into plantlets. As compared to suckers, encapsulated shoot tips present as inexpensive, easier and safer material for germplasm exchange, maintenance and transportation. Table 2.5 showed the effects of different concentrations of sodium alginate ($NaC_6H_7O_6$) and soaking period of calcium chloride dehydrate ($CaCl_2.2H_2O$) on bead formation of lotus explants. Under sterile conditions, green plumules were excised from the seeds and encapsulated using sodium alginate solution for bead formation with ideal texture with uniform, isodiametric shape and size. The optimum concentration for the formation of encapsulation matrix was 3.0% sodium alginate ($NaC_6H_7O_6$). Encapsulated explants were soaked in calcium 100 mM chloride dehydrate ($CaCl_2.2H_2O$) solution for 30 minutes. No suitable beads were formed with low concentration (1-2%) of sodium alginate. Within 10 minutes (Figure 2.8 (a)), soaking in

calcium chloride dehydrate, clear and bead formation with no definite shape was observed. While, within 20 minute (Figure 2.8 (b)) in calcium chloride dehydrate, clear beads, solid and round at outside was observed, however inside bead was still in liquid condition. The problem occurred when the beads were cultured on solid MS basal media, the beads became shrunk and explants died. The optimum soaking period was in 30 minutes (Figure 2.8 (c)) of calcium chloride dehydrate with 3% of sodium alginate which formed very hard bead with very perfect round shape.

Table 2.6 showed the effect of storage period on germination rate of *Nelumbo nucifera* Gaertn. with sodium alginate solution (Figure 2.9 (a)) exposed at 4±1°C and frozen whole seeds at -20±1°C for 90 days. Initially, plumule inside the seeds were encapsulated in sodium alginate solution using sterile 5 ml syringe (Figure 2.9 (b)). The beads were then formed in calcium chloride. These beads were placed at 4±1°C under dark condition to avoid shoot elongation. The highest germination rate was on the first day of encapsulated with 100% germinated when cultured on MS basal media (Figure 2.9 (c)). Beads were cracked (Figure 2.9 (d)) and shoots elongated (Figure 2.9 (e)). Germination rate was reduced to 98.73±0.51% after 15 days. After 90 days in storage (Figure 2.9 (f)), germination rate was only at 53.33±1.22%. This resistance to germination is caused by the seedcoat which is almost impermeable to water penetration in extreme weather (-20±1°C). The probable reasons for this survival tactic in the genus *Nelumbo* are the two fold layers that tightly bonded together. The secret for speeding up the germination process is to remove this protective cover without harming the internal seed using chemical solution or physical method to break through the hard nut.

Whilst, after 60 days in storage through frozen whole seeds method (-20±1°C), the germination rate were remain the highest (100%). After 90 days in storage, the germination rate was still high (93.32±0.63%). According to Kim et al. (2006), after breaking dormancy at 4±1ºC for 6 week, most of seedlings from somatic embryos developed into healthy plants in culture room at 24±1°C.

The development of artificial seed production is effective and acts as an important alternative method of propagation in several commercially important plant species with high commercial values. Synthetic seed production has many advantages in storage over conventional propagation. Consequently, genetic uniformity and stability of the plant could be maintained due to the sterility. As the result, plants could be produced in large scale with high volumes. At the same time, cost would be cheaper. Synthetic seeds technology had been studied on lotus petiole from immature seeds (green) to ensure the viability of lotus propagules. Propagules could be stored atlow temperature (4 ±1°C), for up to 6 months. After 3 months of storage, there were no spoilt propagules, and 94% of total propagules maintained after planting. Water retention and shelf-life of micro shoots were similar among all treatments during storage. The synthetic seeds were inoculated onto MS basal media for germination. The synthetic seeds technology have a high potential to provide an efficient mean to micropropagate nelumbo with elite genotype as well as an alternative way to regenerate nelumbo. The highest frequency of shoot emergence and maximum number of shoots were recorded for beads encapsulated in 3% (w/v) sodium alginate .

In the present work, synthetic seeds were successfully in multiple shoot induction by encapsulating the petioles excised from green seeds and were encapsulated

in 3% (w/v) sodium alginate in MS basal medium with 100 mM $CaCl_2.2H_2O$ as complexing agent. The beads formed using the sterile 3 ml syringe were firmed, oval and isodiametric in shapes. The beads formed were 5 mm in diameter with spherical shape. Encapsulation of *in vitro* derived shoot tips of *Nelumbo nucifera* in different concentrations of sodium alginate dissolved in MS basal medium.

Multiplication in lotus is rarely reported possibly because its recalcitrance to regeneration *in vitro* (Zhao 1999). Many technologies have been developed for lotus production to obtain maximum yield for economical purposes, including discarding the terminal buds of main stock stem (Wen, 1987). Terminal buds were more efficient than axillary buds for shoot induction (Luo et al., 2004a). Plant tissue culture techniques have been developed to efficiently propagate many horticultural crops. Although information is limited to indicate that aquatic angiosperms are adaptable to *in vitro* culture, both direct and indirect shoot organogenesis has been demonstrated in several aquatic plant species (Kane et al., 1990, 1991). Tissue culture of lotus has been reported by researchers mainly in China, Japan and Thailand for the potential and as an alternative approach for lotus propagation. To this date, none report from Malaysia.

According to Ke et al. (1987a) and Liu (1948), only callus induced from immature embryos, green plumule and young cotyledons, while shoots directly induced from plumule. Indirect regeneration of lotus with callus induced from buds, cotyledons and young leaf explants on Murashige and skoog medium (1962), and somatic embryos were successfully induced from callus (Arunyanart and Chaitrayagun, 2005). Contrary from these, in the present research complete direct regeneration successfully achieved from juvenile shoots from both green and yellow seed, within 6 months. A few factors

that need to be considered in selection of explants in tissue culture are source of explants (intact oraseptic plant), explants size, ontogeny, age of explants or the maturation of plant stock (Murashige, 1974a). Explants consist of young cells and tissues normally show better results compared to the old ones. This is because, young meristematic cells divide actively and can respond better to the controlled environment.

Shoots and plantlets were successfully obtained through stem culture (Yamamoto and Matsumoto, 1986, 1988; He and Liu, 1987). Each desired cultural effect has its own unique requirements such as cytokinin (high-cytokinin-low-auxin ratio) for initiation and development of adventitious shoots and auxin for induction of adventitious roots In order to enhance the regeneration rate, MS media was supplemented with NAA and BAP to induce both shoot and root formations. Auxins are well known with their functions in the establishment and maintenance of polarity in organized tissues, and in whole plants their most marked effect is the maintenance of apical dominance and mediation of tropisms.

In plant tissue culture, depending on hormones present in the medium, changes in auxin concentrations may change the type of growth. Table 3.1 showed the response of juvenile shoots from green and yellow seeds on 30 different combinations and concentrations of BAP and NAA of solid MS media. For green seed explants, shoots elongated (Figure 3.2 (a)) within 2 weeks. Rolled leaf (Figure 3.2 (b)) formed in week 12, unrolled leaf (Figure 3.2 (c)) after another 4 weeks. The highest number of shoots per explant for green seeds were on MS media supplemented with combinations of 1.5 mg/l BAP and 0.5 mg/l NAA with 10.33 ±0.23 shoots per explant (Figure 3.2 (d)). The lowest number of shoots per explant for green seeds were on MS media supplemented

with combinations of 3.0 mg/l BAP and 2.5 mg/l NAA with 1.33 ±0.23 shoots per explants, slightly lower than in MS without hormones (1.43 ±0.10 shoots per explants).

Only four treatments were successful for root formation (Figure 3.1 (b)). The highest number of roots per explant for green seeds explants was on MS basal media with 4.33 ±0.53 shoots per explant. MS supplemented with 1.5 mg/l BAP 0.5 mg/l NAA, 1.5 mg/l BAP 1.5 mg/l NAA and 1.0 mg/l BAP 2.5 mg/l NAA gave 3.67 ±0.32 roots per explant, 0.67±0.09 roots per explant and 0.57±0.35 roots per explant, respectively. Pierik (1987) reported, root formation occured with addition of auxin (IAA, IBA, NAA, 2,4-D) at lower concentration, while at higher concentration of cytokinin (BAP, Kinetin, zeatin, 2iP) will induce the formation of shoots. The ratio between auxin and cytokinin will determine the organ formation (Skoog and Tsui, 1948; Miller and Skoog, 1953; Paulet, 1965; Gautheret, 1959). From the results obtained, some formation of abnormal (red and oval) shoots occurred in MS media supplemented with combinations of 1.0 mg/l BAP and 2.5 mg/l NAA, 2.5 mg/l BAP and 2.5 mg/l NAA, 1.5 mg/l BAP and 2.0 mg/l NAA and 1.5 mg/l BAP and 2.5 mg/l NAA (Figure 3.4).

Plant tissue culture system is unique, cytokinins are commonly used in adventitious shoot bud formation, multiple shoots proliferation, somatic embryogenesis and inhibition of root formation. Cytokinins are generally considered as a critical factor for *in vitro* shoot production and there are many reports that BAP exhibits beneficial effect over other cytokinins for shoot multiplication (Dantu and Bhojwani 1987, Rao and Purohit 2006). In intact plants, they particularly stimulate protein synthesis and

participate in cell cycle control. Added to shoot culture media, these compounds overcome apical dominance and release lateral buds from dormancy.

Auxins, together with cytokinins, are involved in controlling morphogenesis in plant tissue culture. Different concentrations and combinations of auxins and cytokinins have different effects on the growth of explants. A balance between auxin and cytokinin growth regulators is most often required for the formation of adventitious shoot and root meristems. The requisite concentration of each type of hormones differs greatly according to the kind of plant being cultured, the cultural conditions and the types of hormones used; interactions between the two classes are often complex, and more than one combination of substances is likely to produce optimal results. A low concentration of auxin is often beneficial in conjuction with high level of cytokinin when shoot multiplication is required. A low concentration of cytokinin (typically 0.5-2.5 μM) is often added to media containing relatively high concentration of auxin for the induction of embryogenic callus, especially in broad-leafed plants (George., 1993).

Media that rich in nutrients such as Murashige and Skoog (1962), were shown to promote vitrification in some plant species (Paques 1991). MS media is the most suitable and most commonly used basic tissue culture medium for plant regeneration from tissues and callus. Generally, when the concentration of auxin is low, root initiation is favored; whereas when the concentration is high, callus formation occurs. The most common synthetic auxins used in tissue culture are 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), and 4-amino-3,5,6-trichloro-2-

pyridinecarboxylic acid (picloram, PIC). Naturally existing indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) are also frequently used.

Table 3.1 showed the responses and comparison on 30 different combinations and concentrations of BAP and NAA on juvenile shoots from green and yellow seeds. The highest number of shoots per explant from yellow seeds were on MS media supplemented with combinations of 0.5 mg/l BAP and 1.5 mg/l NAA (16.00±0.30 shoots per explants). While, no roots formation in all treatments from yellow seed explants (Figure 3.3 (d)) . Difficulties in the establishment of axenic aquatic plant cultures have been reported by Francko (1986a). Within the same period (1-24 weeks) of culture on solid MS media, germination from green seeds were observed exactly the same as mother plants with shoots and roots (complete plantlet). Whilst, regeneration from yellow seeds were different with smaller shoots elongated. No abnormal shoots formation obtained in the treatment of yellow seed explants. In some plant species, MS media was shown to promote vitrification (Paques and Boxus, 1987).

There are many factors governing the success of *in vitro* regeneration, such as the cultivar differences (genetic composition), the physiological status of explants, developmental stages of explants and plant growth regulators added to the media (Nhut et al., 2007). Explants taken from juvenile plant tissue, particularly those of seedlings are usually highly responsive. This can be a genuine effect of material age, but usually reflects the juvenile state of seedling tissues.

Table 3.2 showed the response of i*n vitro* development of shoots in different strength of solid MS media. The results showed that explants from green seeds gave better reponse to higher MS strength. The highest (19.03±0.05) shoots per explants from green seeds was in 8.8mg/l MS powder (double strength). This followed by 6.6 mg/l MS powder (one and a half strength) with mean of 16.01±10.82 shoots per explant. The lowest (1.43 ±0.10) shoot per explant was in 4.4 mg/l MS powder (single strength that formulated by Murashige and Skoog). Whilst, explants from yellow seeds gave the best result (16.06±0.06) shoots per explants in 4.4 mg/l MS powder and not response to others (lower and higher) MS strength.

The pH-dependent and C-assimilation curves in sections of plants grown in acidic and alkaline liquid media were statistically similar (Francko, 1986b). In all cases, the slightly acidic pH values appeared to be preferred for growth and development. Soft and spongy callus was produced at pH below 5.4 and above 8.0 (Chu, 1966). Table 3.3 showed the effect of different pH media in regeneration of lotus shoots from juvenile shoots from green seed and yellow seed explants. From the results, pH range from 4.5-6.5 produced vigorous growth of regeneration of shoots for both green seed explants and yellow seed explants. Media with pH 5.5 resulted in the highest height of shoots for green seed explants with mean of 12.04±0.7 mm and 16.03±0.30 mm for yellow seed explants. These were followed by MS media with pH 4.5 with mean of 10.11±0.5 mm for green seed explants and 13.50±0.41 mm for yellow seed explants. The lowest were in pH 8.5 with mean of 6.75±1.11 mm and 3.44±0.21 mm for green seed explants and yellow seed explants, respectively. Explants responded better in acidic media compared to alkaline media. Therefore, pH 5.5 was suitable for both explants green and yellow seeds compared to normal pH (5.8).

According to Meyer (1930), dormancy and growth of *Nelumbo* was largely influenced by pH, temperature and soil types. Root formation from excised potato buds was maximal at pH 5.7 and evidently inhibited at pH below 4.8 or above 6.2 (Mellor and Stace-Smith, 1969). Nautilocalyx leaves explants showed a rapid and good root formation at pH 5.0 to 6.3 (Vennerloo, 1976). Somatic embryos were developed from excised zygotic embryos of carrot at pH 5.7 in an auxin-free medium (Smith and Krikorlan, 1990). Growth of *Platycerium* leaves sections, sugarcane suspension cells and pineapple stem-tip explants was optimized at pH 5.2, 5.5 and 5.7, respectively (El-Zifza®, 1998; Aydieh et al., 1999; Ebrahim et al., 1999).

Although there are a lot of reports on effects of photo period and temperature on plant growth *in situ*, studies for their effects on *in vitro* plant growth are still very limited. In the present study showed the optimum temperature was 25±1°C with 16 hours photoperiod. Phytochrome played an important role in photoperiodic response of rhizome growth (Masuda et al., 2007): rhizome enlargement occurred under 8 to 12 hour photoperiod while rhizome elongated under a 13 to 14 hour photoperiod. Optimal temperatures are 22 to 32 $^{\circ}$C for lotus growth (Yang et al., 2006).

Table 3.4 showed the effects of distance of the light source in multiplication of lotus shoots. The results showed that the highest height of shoots from green seeds was 9.41±1.11 mm in 250.00 mm light distance. The lowest (1.21±1.01 mm) height of shoot from green seed was in distance of 50.00 mm. While, the highest value for shoot height was 16.67±0.23 mm for yellow seeds in 200.00 mm light distance. From this experiment, light distance of 200.00-250.00 mm was optimum for growth of shoots from green seeds and 100.00-200.00 mm from yellow seeds.

When *in vitro* plantlets were subcultured every 30 days the number of plants remarkably increased within 90 days of culture (Kakuyama and Ogawa, 1997). Table 3.5 showed the response and development of rooting of lotus shoots from green and yellow seeds when subcultured in root formation media. Shoots of about 15-25 mm in height were detached from shoot clumps (Figure 3.6) and transferred to root formation media. Solid MS basal media was identified as the optimum rooting media for green seed explants with mean of 4.33±0.53 roots per explant (Figure 3.5 (b)) . However, the shoots were stunted with slow growth until maximum height of 30.00 mm for 24 weeks (Figure 3.5 (a)). Slightly lower with 3.67 ±0.32 roots per explant for green seed explants on solid MS media supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA. Root formation on solid MS basal media were longer and thicker compared to solid MS media supplemented with BAP and NAA (Table 3.5).

No root formation from yellow seed explants. Alternatively, layered multiple shoots were transferred to solid MS media after 24 weeks on solid MS media supplemented with 0.5 mg/l BAP and 1.5 mg/l NAA (Figure 3.7). Within 4 weeks, primary roots only occurred. While, for green seed explants, primary and secondary roots occurred.

Production of ornamental lotus are at a very small scale in the world and plants are usually sold using dormant rhizomes. In this research, juvenile shoot explants of *Nelumbo nucifera* Gaertn. succesfully established *in vitro.* Stock plant cultures were

increased by repeatedly subcultured at 21-day intervals. Plants cultured in solid MS media produced leaves with elongated petioles.

In natural habitat, the growth of lotus is affected by water level and its fluctuation. The deepest water level recorded was about 2-3 m for wild lotus (Unni, 1971ab, 1976; Kunii and Maeda, 1982; Wang and Zhang, 2004). Rhizomes of some species were killed by anaerobic incubation at 22°C for 7 days, while others could survive and showed normal shoot extention on return to aerobic conditions (Barclay and Crawford, 1982). According to Nohara and Kimura (1997), the maximum depth for lotus growth is 2.4m water depth in artifial environment of concrete ponds, no petiole elongate from 3-5m water depth.

Table 3.6 showed the effects of solid and liquid level of media in regeneration of explants from green and yellow seeds. As control (Figure 3.8(a)), explants were cultured on solid MS basal media. Green shoots elongated with mean of 4.31±0.80 number of shoots per explant and formation of primary root (Figure 3.8(b)). While, yellow shoots turned to green with mean of 9.10±0.51 number of shoots per explant and formation of primary roots. The highest number of shoots per explant was both in ratio liquid to solid 2:1 with mean of 16.67±0.23 number of shoots per explant with formation of primary and secondary roots for explants from yellow seeds (Figure 3.8(c)), while mean 9.00±0.15 number of shoots per explants for green seeds with formation of layered multiple shoots.In ratio liquid to solid with 1:1, both shoots elongated normally with mean 8.33±0.23 and 15.67±0.09 number of shoots per explants for green and yellow shoots, respectively (Figure 3.8(d)).

Even though lotus is an aquatic plant, in ratio of liquid to solid 3:1 (flooded), both shoots turned to brown with mean of 5.33±0.23 number of shoots per explant fromgreen shoots and 10.33±0.23 number of shoots per explant for yellow shoots. The better contact between explants and liquid medium which increases the availability of cytokinin and the ability for nutrient uptake (Debergh, 1983), dilution of any exudates from explants in the liquid medium (Ziv and Halevy,1983) and more adequate aeration in the liquid medium, which enhances growth and multiplication (Ibrahim, 1994).

Figure 3.9 showed the best explants (juvenile shoot excised from yellow seed) regenerated on optimum solid MS media supplemented with 0.5 mg/l BAP and 1.5 mg/l NAA for 20 weeks, developed with new characteristics (layered multiple shoots). A small percentage of *in vitro* plants showed morphological and cytological changes that are termed as somaclonal variation (Scowcroft, 1981; Evans, 1986). This proved that *in vitro* plantlets could not guarantee the new plant produced through *in vitro* multiplication is true-to-type to the mother plant in genotype aspect. The nature of abnormal morphogenesis *in vitro* emphasizes the need for the optimization of plant culture conditions (Gaspar et al., 1987).Tetraploid lotus (4n=32) was produced through *in vitro* culture with colchicine treatment (Yamamoto and Matsumoto, 1990).

Mammals including humans cannot synthesize carotenoids even though they are essential source of retinoids and vitamin A (Botella-Pavia et al., 2004) and Recommended Dietary Allowance (RDA) for 6 mg β-carotene, per day. Humans normally obtain preformed vitamin A (esters of retinols) from animal tissues in the diet and provitamin A carotenoids (mainly β-carotene) from fruits, vegetables and oils

(Allen and Haskell, 2002). Vitamin A supplementation has been extensively researched both in terms of its health and nutritional impact (Sommer and West, 1996). It would be desirable to meet the daily requirements for vitamin A by raising the carotenoid levels within staple foods through genetic manipulation.

Currently there is considerable interest of manipulating carotenoid content and composition in plants to improve the agronomic and nutritional value for human and animal consumption (Cunningham and Gantt, 1998). Therefore, improving nutritional quality of food crops and its ingredients for human consumption is one of the urgent health issues and high priority areas of research worldwide (Hui and Khacatourians, 1995; DellaPenna, 1999).

Colorants are often added in food to enhance its visual aesthetics and to promote sales (Huang et al., 2002). Although the allowable amount of synthetic colorants is reduced for consumer healthiness reasons in recent years, many kinds of synthetic food dyes are still widely used all over the world due to their low price, high effectiveness and excellent stability (Sadecka and Polonsky, 2000).

Generally, synthetic colorants can be classified into water-soluble and fat-soluble colorants based on their solubility. Most fat-soluble synthetic colorants present in the market are azocompounds, such as Sudan I, Sudan II, Sudan III, and Sudan IV. The genetic toxicity of some azo-dyes has been confirmed (IARC, 1975; Calbiani et al., 2004) and structure–activity relationships have been assessed (Searly, 1976; Prival et al., 1988). It is well known that Sudans (I–IV) have been classified as category 3

carcinogen to humans by International Agency for Research on Cancer (Tateo and Bononi, 2004), and the use of Sudan I in foodstuff is forbidden in global food regulation act (DiDonna et al., 2004). However, Sudan dyes were still found in food products exported in European countries (Calbiani et al., 2004).

Regardless of the type of colorants, the pigments are incorporated either under their natural occurrence or under a chemical modified form. As a consequence of these additional pigment needs, the demand in isolated natural colorants has increased as compared with synthetic dyes (Pszczola, 1998; Joppen, 2003). However, this need cannot always be satisfied due to the limitation in the supply of raw materials because the production of pigments using conventional plant cultivation methods is influenced by climatic conditions, plant cultivars and varieties (Rodriguez-Amaya, 2000).

So far, there is no other detail information about the carotenoids and anthocyanins pigments in petals and stigma of lotus except the studies carried out by Rahman et al. (1962), Masato et al. (2002) and Yang et al. (2009). The former work was a preliminary study performed by adsorption chromatography on magnesium trisilicate and just deduced one flavonol. The other one revealed five anthocyanins, but lacked of data to validate them.

The latter only reported on anthocyanins and flavanols in petals of lotus and not the carotenoids and stamens which the most pigmented and yellowish. Thus, there is a solid need to investigate the potential of natural pigments particularly anthocyanins and

carotenoids in lotus flower parts to be fully utilized and commercialized especially in health advantages, food products and dye technology.

Figure 4.1 (a) showed 100g fresh yellow stamen of lotus collected from Tasik Chini, Pahang to prepare 10 g of dried ground sample (Figure 4.1 (b)). Bright and clear yellow supernatant in acetone solution (Figure 4.1 (e)) was stained on cotton (Figure 4.1 (c)). Dried residue with grey colour (Figure 4.1 (d)).Table 4.1 and figure 4.2 showed the effects of different pH on bright and clear yellow supernatant in acetone solution of lotus stamen. At acidic condition (pH 1-6), yellow pigment changed to the darker colour, yellow-brown. While, at strong alkaline condition (pH 12-14), the colour changed to yellow-green. The optimum yellow colour was at pH 8-10.

Table 4.2 showed that the highest total carotenoid content (258.95 μg/g DW) detected in sample 1 and in contrast, the lowest total carotenoid concentration was found in sample 2 (116.29 μg/g DW). Carotenoid analysis performed by HPLC system detected at least three major carotenoid peaks: neoxanthin, unknown and β-carotene (Figure 4.3). The ranges in content for all individual carotenoid pigments among all samples studied were shown in Table 4.2. As shown in Figure 4.3 and Table 4.2, beta-carotene was found substantially higher compared to neoxanthin and unknown carotenoid. Violaxanthin, zeaxanthin and lutein were not found in any of the samples analyzed.

Lotus flower (stamen and stigma) was found to have the total carotenoid content of 526.96 ± 0.52 μg/g DW, whereas for individual carotenoid β-carotene (460 ± 10.28 μg/g DW) was found with a relatively high concentration and neoxanthin (39.26 ± 0.82 μg/g DW) was found in lower concentrations. One unknown Carotenoid also was

detected. Total carotenoid concentration was determined by spectrophotometry and was measured at three different wavelengths: λ 480 nm, 648 nm and 666 nm using Shimadzu UV-1650 PC spectrophotometer as described by Britton et al. (1995) and Othman (2009). This study established that the main carotenoids identified in yellow stamen of lotus are beta-carotene followed by 2 unknown carotenoids.

Detection of individual carotenoid was made at the wavelengths of maximum absorption of the carotenoids in the mobile phase: neoxanthin (438 nm), violaxanthin (441 nm), lutein (447 nm), zeaxanthin (452 nm) and β-carotene (454 nm). The total and individual carotenoid concentration was expressed in terms of microgram per 1.0 g dry weight or freeze-dried matter (μg/g DW). Biological samples were prepared in triplicate and each biological sample was further analysed in triplicate. All manipulations were performed on ice and under subdued artificial light conditions with headspaces of containers flushed with oxygen free nitrogen to help prevent carotenoid degradation.

Carotenoids prone to oxidation and the colour will degrade when exposed to light and heat. Therefore all manipulations were performed on ice and under subdued artificial light conditions with headspaces of containers flushed with oxygen free nitrogen to help prevent carotenoid degradation. Application of carotenoids particularly from *Nelumbo nucifera* Gaertn. as natural colorant which is non-toxic and eco-friendly is still new in this field as well as in coatings and paintings industry. Consequently part of plant pigment research is oriented in finding new sources of pigments. This quest is not only directed in finding natural alternatives for synthetic dyes, but also with the aim to discover new taxons and new procedures for the pigment production, for instance from cell and/or tissue cultures.

Until to date, there is no detail information about the carotenoids pigments in stamens which gave the most pigmented and yellowish in colour. Thus, there is a solid need to investigate the potential of natural pigments particularly carotenoids in lotus flower parts to be fully utilised and commersialised especially in health advantages, food products and dye technology.

Table 4.3 showed the ratio dried stamen sample to volume of 70% (v/v) acetone solution. The best bright yellow colour was from 1.0g/20.0ml (w/v) extraction. In 1.0g/10.0ml (w/v) extraction, the stamen sample was not dissolved. While in 1.0g/30.0ml (w/v) extraction, the yellow colour was not too strong. Table 4.4 showed the glossiness and ratio of pigment and resin in coating process. The highest glosiness was in 1.0ml/30.0ml (v/v) of pigment-resin solution with mean of 74.67±0.33. These followed by 1.0ml/20.0ml (v/v) of pigment-resin solution with mean of 67.01±0.58. The lowest glosiness was in 1.0ml/10.0ml (v/v) of pigment-resin solution with mean of 57.67±0.67. However, better bright red colour with smooth coating only occurred in the lowest glosiness pigment-resin solution. Figure 4.5 showed that yellow pigment in 20% PMMA (resin) was as stable as pigment-resin added with 1% tartaric acid. The colour still maintained as original. However, pigment-resin that added with 1% citric acid faded the colour.

Lotus is an important aquatic economic plant, not only as a dainty and ornamental flower but also as a source of herbal medicine with strong bioactive ingredients such as alkaloids, flavonoids, antioxidants, antisteroids, antipyretic, anticancerous, antiviral and anti obesity properties (Mukherjee et al., 1997; Sinha et al., 2000; Qian, 2002; Sridhar and Bhat, 2007). Plants also are rich in a wide variety of

secondary metabolites polyphenols, such as tannins, terpenoids, alkaloids, and flavonoids, which have been demonstrated to have *in vitro* antimicrobial properties (Gonzalez-Lamothe, et al., 2009).

The variety of compounds produced in *in vivo* and *in vitro* grown plants can show different bioactivity potentials and this is similar with the bioactivities, it will differ between *in vitro* and *in vivo* grown plants. Many studies indicated that only polar extracts of plant showed effective antioxidant activity, and some researchers further proved the moderate polarity extracts are more potent, even if their total antioxidant recovery from the plant is not high. Rapid increase in obesity and dietary-related chronic disease in urban residents resulted from fat in food.

The results revealed that hydroethanolic extracts of rolled leaves from *Nelumbo nucifera* Gaertn., only effective on fungal (*Fusarium* sp and *Trichoderma* sp), while no inhibition zone for tested bacteria. These may be due to photochemical constituents like flavonoid, alkaloids and tannins which also reported by Bose et al., (2007). Antimicrobial activity differ *in vivo* and *in vitro*, probably due to the inherent characteristics of the fully grown plants and the maturity of its chemically active constituents.

Medicinal and herbal plants which contain components of therapeutic have been used as remedies for human diseases for centuries. Plants can produce antifungal compounds to protect themselves from biotic attack that could be essential for fungi infection resistance (Wotjaszek, 1997). Plants also are rich in a wide variety of secondary metabolites polyphenols, such as tannins, terpenoids, alkaloids and

flavonoids, which have been demonstrated to have *in vitro* antimicrobial properties (Gonzalez-Lamothe et al., 2009).

Table 4.5 showed no inhibition zone to all tested bacteria namely; *Staphylococcus aureus, Pseudomonas aeruginosa*, *Bacillus cereus* and *Escherichia coli* (Figure 4.8) for both extraction from *in vivo* and *in vitro* rolled leaves. Table 4.6 showed the highest inhibition zone was from *in vivo* sample for both on *Fusarium* sp. and *Trichoderma* sp. with mean of 9.0±0.1 mm and 7.3±02 mm diametres, respectively. While, inhibition zone for *in vitro* samples were 8 times lower than *in vivo* with mean of 2.0±0.4 mm on *Fusarium* sp. and 1.05±0.3 mm on *Trichoderma* sp..Generally, *in vivo* and *in vitro* rolled leaves extraction inhibited better on *Fusarium* sp. compared to *Trichoderma* sp..

Micropagated plants usually showed low survival rates when acclimatized due to the lost of water, inefficient stomata functions, poorly developed cuticle wax on leaves produced and many others inefficiency. Medium used for transplanting purposes must be sterillized. A chilling treatment before transplanting may improve survival rate and growth of plants. Plantlets transferred directly to greenhouse had 100% mortality, primarily due to poor growth performance (Habib et al., 2001). A gradual acclimatization is a critical step of transplanting and it significantly affects performance of transplanted plants. After being transplanted, *in vitro* plants of lotus were very sensitive to fungi and bacteria, which spread quickly among the plantlets if a nonsterile environment exists. Therefore, individual acclimatization in separate pots or multi-plots was suggested to reduce disease development (Albers and Kunneman, 1992). Low

temperature with high humidity also may benefit transplanting procedure. Plantlets were transferred to sterile clay soil and uncovered in culture rooms for 21 days. Micropropagated plants need sometimes for adaptatation to the environment, so the culture vessels need to be opened a few days before *in vitro* plantlets are transferred to the natural environment.

According to He (2006), it was necessary to treat plantlets 2–3 months at 2–4ºC before transplanting to improve survival rate. The performance of transplanted plants largely depends on acclimatization procedures involving adaptation of plantlets to *ex vitro* conditions of significantly lower relative humidity and higher light intensity. During *in vitro* culture, plantlets grow in high humidity and low irradiance and plants that develop under lower relative humidity have fewer transpiration and translocation problems *ex vitro* and persistent leaves that look like normal ones. After transplanting (acclimatization), about 75% of plantlets survived (Liu et al., 2002; Luo et al., 2004b).

Table 5.1 showed 8 types of soil collected from the original habitats of samples with the pH and survival rates of the plantlets.. The pH ranged from 3-7. The most acidic soils were collected from Tasik Chini with mean pH of 3.97±0.02 with the lowest survival rate (31.34±0.77%) for green seeds and 11.11±0.51% for yellow seeds, while, black mud, white sand and black burn soil were had pH of 4.26±0.12, 4.57±0.13 and 4.89±0.11, respectively. The less acidic soil were in red soil or laterite with mean pH of 6.38±0.27, followed by black clay loam with mean pH of 6.03±0.29, black topsoil with mean pH of 5.14±0.12 and black soil with mean pH of 5.08±0.18. The highest survival rate was in black clay loam for both plantlets from green and yellow seeds with 83.01±0.23% and 69.22±0.43%, respectively. Soil samples collected from Tasik Chini

with mean pH of 3.97±0.02, while the optimum survival for acclimatization plantlets was on black clay loam (mean pH 6.03±0.29).

This recycled clay loam soil is specially prepared containing fertilizers (NPK ,Magnesium, Calcium, TE)) which can last for 3-6 months in water. This heavy soil is being treated against high acidity and any harmful bacteria. Loam soil is much better than sandy soil (Meyer, 1930) for lotus nutrition. According to Wang and Zhan (2004), studies by Wuhan East Lake Flower and Bonsai Research Institute reported that lake soil (pH=6.5) is better than mountain mud (pH=5.6) and garbage soil (pH=7.4). Lotus can tolerate pH 4.5 to 9.0 (Meyer, 1930).

Lotus is a fertilizer-consuming plant. Extra care for transplanting plantlets, since the plants are so fragile and sensitive. The ideal temperature for growing the Indian Lotus is between 20°C and 35°C. *Nelumbo nucifera* is an aquatic plant with horizontal slender, vegetative stolon and thickened storage tuber. It consist of submerged and emerged petioles and two different forms of leaf (lamina), floating and emergent leaf (heterophylly). Heterophylly (more than one leaf form) is common among aquatic plants (Arber, 1920; Sculthorpe, 1967). The leaves float on the water surface at the immature stage and later were raised higher (emerged). At both shallow and deep lakes, lotus is predominant over other submerged and floating-leaved plants because it can shade the other aquatic plants.

Diaphragmatic nets in lotus rhizomes could prevent internal flooding in water depths up to 2 m, which corresponds approximately to the depths at which natural

stands of the lotus growth (Blaylock and Seymour, 2000). *Nelumbo* is also considered to be an invasive plant or weed in some countries (Lancar and Krake, 2002) possibly based on its high productivity, fast growth, and suppression of other aquatic species during competition. *Nelumbo* was competitively dominant in low salinity, fertile conditions, while *Potamogeton* and *Nymphaea* exhibited the greatest salinity tolerance and germination potential. Both the intensity and importance of competition of *N. lutea* were high within low-salinity, fertile conditions, but decreased as salt and nutrient stress increased (Adair, 1997).

The unique adaptation to the deeper water level is through the elongation of the petioles from underwater to the water surface (emerged and submerged). Since the floating leaves were supported by water, the physical strength of petioles could be minimized. According to Ridge (1987), a number of species in which the petioles could elongate to 4-6 times longer under water than in air. The petiole elongation influenced by the water depth (Sculthorpe, 1967; Wooten, 1986) and up to 25 cm per day at 2.4m water depth (Nohara and Kimura 1997). Most petioles are found to reach the full length within 2 weeks after unrolling (Nohara and Tsuchiya, 1990).

Figure 5.4 and Table 5.5 showed development of plantlets transferred to containers (fresh water aquarium and water container). The first 8 months being acclimatized, stem and leaves grew vigorously. Figure 5.7 showed the first four leaves floating on water surface with red spots at the back when transferred to water container (Figure 5.8). The same thing happened when transferred to aquarium (Figure 5.7). However, when the leaves emerged from water surface, leaves became green in colour. The growth of acclimatized plantlets were showed in Table 5.6.

Optimal temperatures are 22 to 32 °C for lotus growth (Yang et al., 2006) and lotus perform better under full sun than in shady places. According to Li et al (2000b), increasing of light intensity improved leaf greenness, stalk thickness and number of flowers in tunnel house for winter production.Total biomass increased significantly with increasing light in a tank experiment, although the survival of *Nelumbo lutea* seedlings was high in all tested light level (Snow, 2000).

Li et al. (1984) exposed plantlets to 3–4 days acclimation under plenty light before being transplanted to neutral soil. Hosoki et al. (1989) placed transplanted plantlets under a plastic cover at 18–20ºC and 16 hour illumination of 52 $\mu EM^{-2} S^{-1}$ cool white fluorescent lamps. Beruto et al. (2004) reported that a gradual acclimatization of plantlets in closed containers placed in culture room resulted in good quality plantlets and 80 ± 10% of transplanted plants survived. According to An (2005), increased light exposure of plantlets in the open tubes for 3–4 day before transplanting and used a plastic cover for two weeks to maintain high relative humidity after transplanting. By this acclimation procedure, 80% survival rate has been obtained for *P. rockii*. The information about effect of light on performance of transplanted peony *in vitro* plants is extremely limited. Albers and Kunneman (1992) reported that increased day length (8, 12, 16 h) at a light intensity (45 mmol s^{-1} m^{-2}) during the first 3 week resulted in increase of shoot length and leaf size, but the difference in shoot length disappeared .

Kong and Zhang (1998) reported that low temperature gave high survival rate (36%) of plants at 15–20ºC but only 8 % at 25–30ºC in vermiculite medium. However,

Albers and Kunneman (1992) found no difference in temperature treatments (5, 10, 15ºC) for growth of transplanted plants.

In United States, mass production and high demand of lotus sale in container promote the water plant nurseries (Creamer, 2008). Ornamental lotuses are usually collected and limitedly produced by the lotus-related research institutions including China Lotus Research Center, Wuhan Botanical Gardens, Nanjing Zhongshan Botanical Gardens, University Of Tokyo and Auburn University in USA. China is considered as the research center of wild *Nelumbo nucifera* and its cultivars (Wang and Zhang, 2004).

The ability of lotus in chromium removal and in bioassay was assessed by Vajpayee et al. (1999) using a few modifiable physiological responses, maximum being in roots. Kumar et al. (2002) reported that the accumulation of Cu and Zn was very high in rhizomes, fruits and carpels of lotus cannot be use as food. Kanabkaew and Puetpaiboon (2004) reported that wastewater treatment system with lotus showed better removal efficiency than a system with *Hydrilla verticillata*. Sun et al. (2000) studied the strong ability of lotus to absorb and accumulate Fe,Mn, Cu, Zn,P b and Cd.

Nelumbo nucifera was used to investigate water quality of reservoirs which was possibly responsible for chronic renal failure in Sri Lankathe (Bandara et al., 2007) Ebrahimpour and Mushrifah (2007) reported that the lowest or second lowest of concentration of heavy metals (Cd, Cu and Pb) acccumulation is in *Nelumbo nucifera*, among five aquatic plants in Malaysia: *Lepironia articulata, Pandanus helicopus, Scirpus grossus, Cabomba furcata and Nelumbo nucifera.* Studies in Kanewal reservoir, Nirmal Kumar et al. (2008) reported that highest average concentration of heavy metals

(Cd, Co, Cu, Ni, Pb, and Zn) is in *Eichhornia crassipes* and the lowest is in *Nelumbo nucifera* among seven species aquatic plants: *Eichhornia crassipes*, *Eichhornia colonum*, *Hydrilla verticillata*, *Ipomoea aquatica*, *Nelumbo nucifera*. According to Li and Sun (2006), lotus that harvested from a campus pond contained high concentration of six heavy metals (Pb, Cd, zn, Cu, Mn and Cr) from the laboratory wastes.

Until now, unsuccessful regeneration from flower lotus has been reported by Arunyanart (1998) and Arunyanart and Chaitrayagun (2005). Only in 2008, Shou et al reported the *in vitro* multiplication of the economically valuable vegetable lotus through shoot proliferation from underground rhizomes.

In the present study, direct regeneration of *Nelumbo nucifera* Gaertn. were successfully achieved from green seed explants cultured on solid MS media supplemented with combinations of 1.5 mg/l BAP and 0.5 mg/l NAA with 10.33 ±0.23 shoots per explant (true-to-type), and with 3.67 ±0.31 roots per explant. Whilst, direct regeneration of *Nelumbo nucifera* Gaertn. were successfully achieved from yellow seed explant cultured on solid MS media supplemented with combinations of 0.5 mg/l BAP and 1.5 mg/l NAA with 16.00±0.30 shoots per explant, with new characteristics of layered multiple shoots. Roots formed on solid MS basal media.

CHAPTER 7

CONCLUSION

In chapter 2, 3 and 5, initial study was about the effects of pH on samples of *Nelumbo nucifera* Gaertn.. pH 5.5 was recognised as optimum pH for *in vitro* germination of three types of seeds according to the maturity (black seeds, green seeds, yellow seeds) and *in vitro* regeneration from both green and yellow seeds. For *in vitro* germination, the highest shoot length was on solid MS media with pH 5.5 for black seeds, green seeds and yellow seeds (Figure 2.5), with mean of 64.03±0.02 mm, 32.55±0 mm and 11.11±0.03 mm, respectively. For *in vitro* regeneration through tissue culture system, solid MS media with pH 5.5 resulted in the highest height of shoots for green seed explants with mean of 12.04±0.7 mm and 16.03±0.30 mm for yellow seeds. In acclimatization treatments, the most acidic soils were collected from Tasik Chini with mean pH of 3.97±0.02 and with the lowest survival rate, 31.34±0.77% for green seeds and 11.11±0.51% for yellow seeds . The highest survival rate was in black clay loam (mean pH 6.03±0.29) for both plantlets from green and yellow seeds with 83.01±0.23% and 69.22±0.43%, respectively.

The present study proved that explants developed the best regeneration on solid MS media compared to liquid media, eventhough, lotuses were known as fresh water plant. *In vitro* germination and regeneration were successfully achieved on solid MS

media for shooting and rooting with the highest shoot length for seeds without cotyledons (Figure 2.4 (f)) with 28.00±0.55 mm for black seeds, 26.00±0.16 mm for green seeds and 14.43±0.09 mm for yellow seeds. Primary root induction was obtained on solid MS basal media after 12 weeks in culture. All type of seeds responded to the media with 1 mm root elongation per day. The maximum root length for both black and green seeds were 14 mm on the 14^{th} day with 3 green shoots and 6 white primary roots. While, maximum root length for yellow seeds were 17 mm on the 18^{th} day with 3 green shoots and 6 white primary roots. Solid MS basal media was identified as the optimum rooting media for green seeds with mean of4.33±0.53 roots per explant (Figure 3.5 (b)). However, the shoots were stunted with slow growth until maximum height of 30.00 mm for 24 weeks (Figure 3.5 (a)). Slightly lower (3.67 ±0.32) roots per explant from green seeds on solid MS media supplemented with 1.5 mg/l BAP and 0.5mg/l NAA. Roots formations on solid MS basal media were longer and thicker compared to solid MS media supplemented with BAP and NAA.

Encapsulated (synthetic seeds) and frozen seeds of *Nelumbo nucifera* Gaertn., can be used as an alternative storage for micropropagation since this species is one of the endangered species in Asia, Australia and North America. Juvenile shoots from lotus flower encapsulated in 3.0% of sodium alginate ($NaC_6H_7O_6$) as the encapsulation matrix, then soaked for 30 minutes in 100mM calcium chloride dehydrate ($CaCl_2.H_2O$) as the complexing agent. This synthetic seeds are stored and preserved at low temperatures (4±1°C and -20±1°C) and dark condition (covered with aluminium foil), ready for germination when necessary. These seeds have potential as an easy transfering and long term storage and maintaining the viability of the clones. This advantages could expand the species for another experiment or research (ready-seed). The highest germination rate was on the first day of encapsulated with 100% germinated cultured on

MS basal media (Figure 2.9 (c)). Beads were cracked (Figure 2.9 (d)) and shoots elongated (Figure 2.9 (e)). Germination rate was reduced to 98.73±0.51% after 15 days. After 90 days in storage (Figure 2.9 (f)), germination rate was only at 53.33±1.22%. Through frozen whole seeds method (-20±1°C), 100% germination rate was achieved until 60 days in storage. After 90 days in storage, the germination rate was still high with a value of 93.32±0.63%.

Plant regeneration with mass and rapid production of multiple shoots has been successfully achieved in *vitro* from juvenile shoot explants. The highest number of shoots per explant for green seeds was on MS media supplemented with combinations of 1.5 mg/l BAP and 0.5 mg/l NAA with a value of 10.33 ±0.23 shoots per explant (Figure 3.2 (d)) and the highest number of roots per explant was on MS basal media with 4.33 ±0.53 shoots per explant. The highest number of shoots per explant for yellow seeds were on MS media supplemented with combinations of 0.5 mg/l BAP and 1.5 mg/l NAA with 16.00±0.30 shoots per explant. Unfortunately, no root formation from yellow seed explants. The layered multiple shoots were transferred to solid MS media after 24 weeks on solid MS media supplemented with 0.5mg/l BAP and 1.5mg/l NAA (Figure 3.7). For maintenance in double layered media, the highest number of shoots per explants was both in the ratio liquid to solid 2:1 with mean of 16.67±0.23 number of shoots per explant with formation of primary and secondary roots for explant from yellow seeds, while mean 9.00±0.15 number of shoots per explants for green seeds with formation of layered multiple shoots.

Pigment detection and extraction from lotus stamen through HPLC analysis system was found to have the total carotenoid content of 526.96 ± 0.52 µg/g DW

whereas for individual Carotenoid β-carotene (460 ± 10.28 µg/g DW) was found with a relatively high concentration and neoxanthin (39.26 ± 0.82 µg/g DW) was found in lower concentrations. The optimum yellow colour was obtained at pH 8-10. The potential of carotenoid extraction with high pharmaceutical values act as nutrious food and colorant, as well as in coating applications. The highest glossiness was in 1.0ml/30.0ml (v/v) of pigment-resin solution with mean of 4.67±0.33. Yellow pigment in 20% PMMA (resin) was as stable as pigment-resin added with 1% tartaric acid, coated on glass slides. While, the addition of 1% citric acid reduced the carotenoid color.

Furthermore, extraction from *in vivo* and *in vitro* rolled leaves were used in determination of antimicrobial activity. The highest inhibition zone was from *in vivo* sample for both on *Fusarium* sp. and *Trichoderma* sp. with mean of 9.0±0.1 mm and 7.3±02 mm diameters, respectively. Inhibition zone for *in vitro* sample were 8 times lower than *in vivo* with mean value of 2.0±0.4 mm on *Fusarium* sp. and 1.05±0.3 mm on *Trichoderma* sp.. No inhibition zone was seen in all tested bacteria *Staphylococcus aureus, Pseudomonas aeruginosa*, *Bacillus cereus* and *Escherichia coli* for both extractions from *in vivo* and *in vitro* rolled leaves.

This research was completed by the successful acclimatization of *in vitro* plantlets in the green house. 70% of survival plantlets transfered to containers with ½ loam soil added with ¼ of fresh water adapted smoothly to the new environment with fully sun exposure. Based on morphological characteristics observation, lotus that regenerated *in vitro* system from green seed explants were similar to the mother plant (true-to-type) and new characteristics with layer shoots for yellow seed explants were observed.

Until to date, this research reveals two major new findings of *Nelumbo nucifera Gaertn*. as followed:

1. From tissue culture system, new characteristic of layered multiple shoots from explants from yellow seeds were successfully regenerated on solid MS basal media supplemented with 0.5 mg/l BAP and 1.5 mg/l NAA.

2. Through HPLC analysis system, lotus stamen was found to have the total carotenoid content of 526.96 ± 0.52 µg/g DW, whereas for individual Carotenoid β-carotene (460 ± 10.28 µg/g DW) was found with a relatively high concentration and neoxanthin (39.26 ± 0.82 µg/g DW) was found in lower concentrations. One unknown Carotenoid also was detected.

In future, cytological studies of *Nelumbo nucifera* Gaertn. needs to be applied to investigate the cellular behaviour of this fresh water aquatic plant. The studies of mitotic index, ploidy level, chromosome number, cell cycle, nuclear and cell area can determine variations of the plant genotype and phenotype, *in vivo* and in vitro.