ESTABLISHMENT OF MICROPROPAGATION PROTOCOL FOR BANANA (*Musa acuminata* x *M. balbisiana*) CV. PISANG AWAK (ABB GENOME) VIA TEMPORARY IMMERSION SYSTEM

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UNIVERSITI SAINS MALAYSIA
2011

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by

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Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

February 2011

Dedicated to
My Mum, Dad,
Brother & Aunts

ACKNOWLEDGEMENT

I would like to express my heartfelt thanks to my supervisor, Professor Chan Lai Keng and my co-supervisor Dr Sreeramanan Subramanian from the school of Biological Sciences, Universiti Sains Malaysia, Penang. I thank them both for their unceasing support, guidance and invaluable advice to me throughout my studies. For this, I am deeply thankful and grateful.

I appreciated and acknowledge UPEN Kelantan for research funding. I thank the Universiti Sains Malaysia specifically to the Dean of School of Biological Sciences and the Dean of Institute of Higher Learning of Universiti Sains Malaysia for allowing me to pursue my higher degree here. I thank the school of Biological Sciences for the utilisation of facilities.

Special thanks to my lab-mates of whom have helped and supported me through challenges and hardship that I coped during my studies, Fung Hui, Lay Pin, Pey Shan, Poh Liang, Marvin, Chee Leng, Zainah, Kiah Yann, Shu Ying, Novi, Melati, Salmee, Nadia, Song Jin, Eu-Zhin, Farah, Chee Keong, Fariz, Dr Arvind and everyone else who has been part of the team in Plant Tissue and Cell Culture Laboratory.

I would like to take this opportunity to thank my dear family, my father and brother for their unconditional love and support and also my aunts and friends who have supported me in my research work along the way.

Au Vun Hui

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BA	Benzyladenine
IAA	Indole-3-Acetic Acid
IBA	Indole-3-Butyric Acid
MS	Murashige and Skoog (1962)
NAA	α-Naphthalene Acetic Acid
TIS	Temporary Immersion System
RITA®	Automated Temporary Immersion System
APCS	Automated Plant Culture System
BIT®	Twin Flask System
v/v	Volume per Volume
w/v	Weight per Volume

PENUBUHAN PROTOKOL MIKROPROPAGASI PISANG (*Musa acuminata* x *M. balbisiana*) CV. PISANG AWAK (GENOM ABB) DENGAN SISTEM RENDAMAN SEMENTARA

ABSTRAK

Dua protokol pensterilan permukaan untuk mendapat eksplan pucuk aseptik Pisang Awak telah berjaya dikenalpasti bagi tujuan mikropropagasi. Eksplan aseptik daripada sulur yang diperolehi selama satu hari dapat dihasilkan dengan menggunakan kaedah pensterilan permukaan satu peringkat dengan 20% (v/v) Clorox® selama 20 minit. Teknik pensterilan permukaan dua peringkat boleh digunakan bagi sulur yang diperolehi lebih daripada satu hari. Protokol ini melibatkan pensterilan permukaan dengan 20% (v/v) Clorox® selama 20 minit pada peringkat pertama diikuti dengan 10% (v/v) Clorox® selama 10 minit pada peringkat kedua, kemudian diinokulat ke atas medium MS pepejal yang ditambahkan dengan 150 mg/L Kanamycin selama tujuh hari. Proliferasi pucuk dapat dilaksanakan dengan mengkultur eksplan pucuk aseptik yang dibelah dua pada medium MS pepejal atau cecair yang ditambahkan dengan 5.0 mg/L BA untuk merangsang penghasilan pucuk berbilang. Pucuk berbilang ini dipisahkan secara individu dan pengakaran paling baik dilakukan dalam medium MS asas pepejal. Anak benih yang mempunyai ketinggian minimum 2.0 cm dan sistem pengakaran yang berkembang baik dapat diaklimatisasi dengan amat baik di rumah hijau. Aplikasi 0.75 g/L baja NPK (15:15:15) sepanjang proses adaptasi sekunder dapat meningkatkan pertumbuhan anak benih Pisang Awak. Satu protokol propagasi secara besar-besaran yang menggunakan satu modifikasi sistem rendaman sementara (TIS) telah dibentuk. Bilangan anak benih yang optimum dapat diperolehi di dalam TIS dengan menginokulasi lapan pucuk dibelah dua ke dalam 100 mL medium MS yang

ditambahkan dengan 5.0 mg/L BA dan 30 g/L sukrosa pada pH 5.7 serta direndam selama 10 minit setiap 24 jam di bawah keadaan cahaya berterusan. Protokol TIS optimum yang digunakan bagi penghasilan anak benih Pisang Awak secara besar-besaran juga sesuai bagi penghasilan anak benih Pisang Raja. TIS telah dibukti lebih baik daripada medium pepejal dan medium cecair bergoncang berterusan bagi proliferasi pucuk Pisang Awak. Sebanyak 93% pucuk mikro yang berakar daripada TIS dapat diaklimatisasi terus kepada keadaan rumah hijau. Manakala pucuk mikro tidak berakar yang diperolehi daripada medium pepejal dan TIS perlu melalui proses pengakaran dalam medium MS asas dan sebanyak 89 – 92% anak benih dapat hidup selepas diaklimatisasi di rumah hijau.

ESTABLISHMENT OF MICROPROPAGATION PROTOCOL FOR BANANA (*Musa acuminata* x *M. balbisiana*) CV. PISANG AWAK (ABB GENOME) VIA TEMPORARY IMMERSION SYSTEM

ABSTRACT

Two surface-sterilization protocols were established to obtain aseptic shoot explants of Pisang Awak for micropropagation purpose. Aseptic explants could be established from one day old suckers by using one-stage surface-sterilization method with 20% (v/v) Clorox® for 20 minutes. Two-stage surface-sterilization technique could be used for more than one day old suckers. This protocol involved surface-sterilization with 20% (v/v) Clorox® for 20 minutes at the first stage followed by 10% (v/v) Clorox® for 10 minutes at the second stage, then inoculated onto solid MS medium supplemented with 150 mg/L Kanamycin for seven days. Shoot proliferation was achieved by culturing the aseptic half shoot explants onto solid or liquid MS medium supplemented with 5.0 mg/L BA to induce multiple shoots formation. The multiple shoots were separated individually and best rooting could be achieved using solid basic MS medium. Plantlets with a minimum height of 2.0 cm and well developed root system could be acclimatized very well in the greenhouse. Application of 0.75 g/L NPK fertilizer (15:15:15) during secondary hardening process enhanced the growth of Pisang Awak plantlets. A mass propagation protocol using a modified temporary immersion system (TIS) was established. Optimum number of plantlets could be achieved in TIS by inoculating eight half shoots in 100 mL MS medium supplemented with 5.0 mg/L BA and 30 g/L sucrose at pH 5.7 immersed for 10 minutes every 24 hours under continuous light condition. The optimized TIS protocol for the mass production of Pisang Awak plantlets was also suitable for mass production of Pisang Raja plantlets. TIS was proven to be more

efficient than gelled medium and shake flask system for shoot proliferation of Pisang Awak. Rooted micro-shoots established from TIS could be acclimatized directly to greenhouse condition with 93% survival percentage. While micro-shoots without root established from gelled medium and TIS needed to undergo rooting in basic MS medium and 89 - 92% of the plantlets survived after acclimatized in the greenhouse.

1.0 INTRODUCTION

Bananas (*Musa* spp.) are perennial monocotyledons plants from the Musaceae family (Purseglove, 1976). They are planted in more than 120 nations in tropical and subtropical zones on five continents in the world. The products produced from bananas provide a basic food source for million of people in developing countries (Daniels *et al.*, 2002) and have a significant ecological and socioeconomic role (Charrier *et al.*, 2001). Total world productions of bananas and plantains are approximately 100 million tons per year (FAO, 2007).

Banana is one of the major fruit crops planted in Malaysia with annual production of approximately 180,000 tonnes that accounted for more than 15% of the yearly fruit production and more than RM30 million (US$8 million) of balance trade. Total banana cultivated land was more than 11% of the total area occupied by fruit crops (Hassan, 2005). About 50% of the total banana cultivated area is planted with Pisang Cavendish and Pisang Berangan cultivars for both export and local consumption. The remainder popular local cultivars for the domestic market are Pisang Rastali, Pisang Mas, Pisang Awak, Pisang Raja, Pisang Abu, Pisang Nangka and Pisang Tanduk. Bananas are normally eaten fresh, processed or cooked. The export of Malaysian banana is mainly to Singapore, Brunei, Hong Kong and the Middle East. Nevertheless, the banana production in Malaysia has decreased due to the high labor costs, outbreak of diseases and marketing issues (Jamaluddin, 2000).

Musa cv. 'Pisang Awak' (ABB genome) is also known as Ducasse banana in Australia. It is the most common banana in Thailand, and is also common in north-eastern India and Malaysia (Purseglove, 1976). It is one of the most vigorous and hardy cultivar in ABB genome of the Eumusa series of banana cultivars. The fruits

may or may not contain seeds (Stover and Simmonds, 1987). Pisang Awak generally is consumed as fresh or fried fruit. The floral buds of Pisang Awak were used in many dishes preparation in Thailand (Charrier *et al.*, 2001).

Suckers, or pieces of the rhizome are commonly used as planting materials in plantation (Crane and Balerdi, 1998). There are five types of planting material used in plantation: peepers, sword suckers, maiden suckers, bits of large corms and water suckers. The material preferred for propagation differ in different places of the world (Purseglove, 1976). However, the spread of detrimental nematodes, insects, Black Sigatoka and Panama disease by field-grown suckers cause a major problem to conventional propagation in plantation (Roels *et al.*, 2005). Pisang Awak is normally propagated by using suckers only by smaller holders for local consumption as dessert fruit in Malaysia (Jamaluddin, 1994). With the usage of unripe fruits of Pisang Awak for the production of high fiber flour (Chong and Noor Aziah, 2008), this has encouraged commercial planting of this cultivar. This ultimately resulted in shortage of plantlets for large scale planting.

Conventionally bananas are being planted using suckers which are usually slow in growth and cannot provide uniform plantlets at one given time. Mass production of uniform banana plantlets by using tissue culture technique might be an alternative to solve this problem. The benefits of this system are the plantlets produced are uniform, free of most diseases and nematodes (Crane and Balerdi, 1998). However, conventional *in vitro* culture method using gelled medium is very slow and labor intensive resulting in high cost in producing these banana plantlets. Micropropagation using liquid medium is suitable for faster proliferation of plantlets with reduction in production costs but it can result in asphyxia and hyperhydricity in some of the plantlets (Berthouly and Etienne, 2005).

Temporary immersion system (TIS) could be used to eliminate hyperhydricity and asphyxia in plants since explants came into contact with the liquid medium periodically (Berthouly and Etienne, 2005). Preil (2005) reported that TIS ensured maximum growth of plantlets by providing an efficient gaseous exchange, less water retention and better nutrient medium absorption. TIS was found to produce positive result on microtuberization, somatic embryogenesis and shoot proliferation in certain plant species (Berthouly and Etienne, 2005). TIS had been proven to be able to produce healthy sugarcane, pineapple seedlings, potato microtubers and apple rootstock (Lorenzo *et al.*, 1998; Escalona *et al.*, 1999; Jiménez *et al.*, 1999; Zhu *et al.*, 2005). Optimizing immersion time (frequency and duration) with optimized volume of medium is very important for TIS effectiveness in improving shoot proliferation. TIS also enhances plant quality while plantlets produced via TIS could acclimatize better compared to conventional micropropagation methods. In addition, up scaling of shoot proliferation using TIS was found to reduce production cost (Berthouly and Etienne, 2005).

Several authors had reported shoot proliferation of banana via TIS (Alvard *et al.*, 1993; Matsumoto and Brandão, 2002; Colmenares and Gimenez, 2003; Roels *et al.*, 2005). However, the *in vitro* propagation of Pisang Awak using TIS has not been reported. Hence, this research was carried out with the following objectives:

1. To establish a suitable surface-sterilization protocol for obtaining aseptic shoot explants of Pisang Awak.
2. To establish a micropropagation protocol for the production of *in vitro* Pisang Awak plantlets.
3. To obtain an optimum protocol of temporary immersion system (TIS) for mass production of *in vitro* Pisang Awak plantlets.

4. To compare the efficiency of TIS on shoot proliferation of Pisang Awak with shake flask system and gelled medium system.

2.0 LITERATURE REVIEW

2.1 Musaceae

2.1.1 Banana Cultivars

Bananas are the perennial monocotyledons from the *Musa* genus and Musaceae family. They are normally grown in the tropics located at latitude 20° above and below the equator that have broad seasonal variation of temperature and rainfall (Pua, 2007). The genus *Musa* contains about 40 species and is divided into five sections (Eumusa, Rhodochlamys, Callimusa, Australimusa and Incertae sedis) (Purseglove, 1976).

The known wild bananas (*Musa* spp.) are *Musa acuminata* and *Musa balbisiana* (Crane and Balerdi, 1998). The origins of the edible bananas in the Eumusa section are from these two wild species (Stover and Simmonds, 1987). The majority of the commercial banana cultivars are triploids (3n = 3x = 33), originating from the polyploidization and interspecific hybridization of these two wild species with genome of AAA, AAB and ABB (Robinson, 1995). Eumusa is the largest and the most diversified section. They are widely distributed throughout South East Asia from India to the Pasific Islands (Daniels *et al.*, 2001). There are many banana cultivars world wide from the AA, AAA, AAAA, AB, AAB, ABB and ABBB genomes (Simmonds, 1970). In Malaysia, the most common cultivars are Pisang Awak, Pisang Berangan, Pisang Raja and Pisang Rastali.

The scientific name of Pisang Awak is *Musa acuminata* x *M. balbisiana* (ABB genome). It is known as Ducasse banana in Queensland, Australia. It is called as Pisang Klotok in Indonesia and Klue Namwa in Thailand. It is the major banana cultivar in Thailand and also common in north-eastern India and Malaysia. It is either

eaten fresh or cooked (Robinson, 1999). It is one of the most vigorous and hardy bananas among other cultivars. It generally produces a heavy bunch of small fruits which tend to be partially fertile and seedy when cross-pollinated by edible or wild diploids (Purseglove, 1976).

Pisang Berangan is known as *Musa acuminata* (AAA genome) cv. 'Masak Hijau'. It is also called as Pisang Masak Hijau in Malaysia and Jamaican Lacatan in Jamaica. It is the most vigorous cultivar of the Cavendish Subgroup and an important commercial edible banana grown in Puerto Rico, Jamaica and planted with coffee in Ecuador and Colombia (Stover and Simmonds, 1987).

Pisang Raja is *Musa acuminata* x *M. balbisiana* (AAB genome) cv. 'Raja'. It is a popular cultivar in Malaysia and Indonesia but unknown in India and Africa. It is commonly eaten as dessert banana. Its yield inclines to be low as each bunch bears only six to nine hands. It is a vigorous cultivar resistant to leaf spot and Fusarium wilt disease (Robinson, 1999).

Pisang Rastali is *Musa acuminata* x *M. balbisiana* (AAB genome) cv. 'Silk'. It is also known as 'Silk Fig' in West Indies, 'Latundan' in The Philippines and 'Apple' in Hawaii. It is distributed as widely as 'Dwarf Cavendish'. It is moderately hardy and does not bear fruits heavily. It is a well known dessert cultivar in the tropics and it has a white, apple-flavored fruit flesh that must be eaten fully ripe (Robinson, 1999).

2.1.2 Biology of Banana

Banana is a 2 - 9 m tree-like giant perennial and has an underground rhizome. The pseudostem composes of overlapping leaf sheaths with new leaves and the inflorescence emerges through the base of the pseudostem (Purseglove, 1976). The

buds from the corm produce shoots (suckers) and short rhizomes near the parent. Adventitious roots build a dense mat and extend until 4 - 5 m radius and 75 cm deep into the soil from the parent (Nakasone and Paull, 1998). The leaf of banana composes of a stout petiole, a sheath and a lamina. The tight packing of abundance sheaths creates the pseudostem and during its lifetime, one pseudostem may contain more than 40 leaves (Crane and Balerdi, 1998).

After 10 - 15 months of planting, the banana inflorescence (flowering stalk) appears from the center of the pseudostem. Banana flowering process is called shooting. About 10 - 20 flowers emerge spirally along the axis of the inflorescence and cover by purplish-to-greenish fleshy bracts. The first row of flowers is female flowers and is called 'hands', from which the fast growing ovaries develop parthenocarpically (without pollination) into fruit bunches. The last emerged flowers are functionally male and the emerged fruit is a berry (Crane and Balerdi, 1998).

2.1.3 Nutritional Values and Uses of Banana

The pulp of a ripe banana is easily digested and sugar-rich. It contains about 70% water, 27% carbohydrate, 1.2% protein, 0.3% fat, 0.9% ash and 0.5% fibre. The banana is considered a good source of vitamin A, C, B1 and B2 and contains several important minerals, including potassium, calsium, iron and sodium. Carbohydrate contributes about 95% of the total energy of banana while the fat provides only 1 - 2% and protein gives 3% of total energy of banana. Bananas are often used in low fat diets because of their low fat and high carbohydrate contents (Chandler, 1995). Manual labors and sports players who need great amount of glucose are normally given bananas to sustain sufficient levels of muscle action. Bananas also have great values as a staple food, therapeutic and global nutrition (Sharrock and Lusty, 2000).

Bananas are mainly eaten raw or can be used as desserts and ingredients for breads, salads and candy (Crane and Balerdi, 1998). Bananas are easily digested by children and often soft ripe banana is the first solid food used to feed the babies (Sharrock, 1997). Sliced bananas dipped in flour and fried in coconut oil known as 'pisang goreng' is the Malaysian favourite (Piper, 1989). Bananas have been processed to prolong the shelf life to produce many products. For instance, dried chips of unripe fruits are kept as famine food in some parts of Uganda while figs are prepared by drying slices of ripe fruits. Bananas can also be made into flour by drying and grinding the green fruit while grounded powder is prepared from the ripe fruits (Sharrock, 1997). Other products derived from banana are syrup, puree, essence, flakes, juice, jams, jellies, vinegar and alcoholic beverages (Thompson, 1995).

Besides the fruit, other parts of the banana plant are utilized as food. The shoots and corms are consumed as vegetable (Nakasone and Paull, 1998). The male buds, with the outer fibrous bracts removed, are eaten as a boiled vegetable in South-east Asia. After harvest, the banana 'heart' could be taken from centre of the pseudostem for cooking and the taste is similar to bamboo shoots (Sharrock, 1997). The heart of the banana stalk is used for cooking Burmese curries (Piper, 1989). Banana pseudostem is cooked and is canned with tomatoes and potatoes in a curry sauce and consumed as vegetable in India. The grounded, fined, roasted and dried green plantains are used as replacement for coffee (Morton, 1987). While the banana inflorescence is cooked and eaten as a vegetable in Bengal (Singh, 1979). In Thailand, the floral buds of Pisang Awak are used in many cooking preparations (Bakry *et al*., 2001).

Besides being used as food, the green banana leaves are often used as umbrellas, disposable plates and food wrappers. In Southern India, bananas are grown commercially for their leaves and are used as 'biological plate' for serving food. In West Africa, banana leaves act as temporary mats used for cocoa fermentation. Besides, banana leaves are also used as packing materials, thatching and wrapping cigarettes. Fibre from the pseudostems could be made into ropes, strings and threads, papers, baskets, toys, table mats and wall hangings. Banana pseudostems are also used for the preparation of animal feeds to feed the cattles and pigs (Sharrock, 1997). The strips of dried pseudostems are used to make seat pads for benches in Ecuador. The dried banana peels contain 30 - 40% tannin and used to blacken leather while high potash in its ash is used for producing soap. Moreover, the burned peels of unripe fruits for some banana cultivars are used for dyeing. Banana plants can also act as ornamental for home landscape (Crane and Balerdi, 1998).

Every part of the banana plant has medicinal values. The flowers are used for treating dysentery, bronchitis, ulcers and diabetics. The astringent banana sap is used to cure fevers, hemorrhages, epilepsy, hysteria, leprosy, diarrhea and acute dysentery. The astringent ashes of unripe banana peels and leaves are used to treat diarrhea, dysentery and serious ulcers. While the banana roots are used for the treatment of dysentery and digestive problems. Diarrhea and catarrh are treated with banana seed mucilage in India (Morton, 1987). Children who suffered from celiac disease (allergy to carbohydrates) and people with various intestinal problems were fed with bananas to relieve the symptoms (Samson, 1986). The pounded peels of ripe bananas can be wrapped directly around cuts or wounds during emergency as the inside of the peel contains anti-septic properties (Sharrock, 1997).

2.1.4 Propagation of Banana

Bananas can be propagated via sexual and asexual methods. Seeds are only used in sexual propagation for breeding purposes. Most of the important commercial cultivars are female-sterile and propagated asexually (Nakasone and Paull, 1998). There are five types of planting materials used in asexual propagation: peepers, sword suckers, maiden suckers, water suckers and bits of large corms. Peepers are very young suckers emerging from the soil with just scale leaves (Purseglove, 1976). Sword suckers are produced from buds or eyes low on corm and joined to the mother rhizome with narrow sword leaves (Crane and Balerdi, 1998). Maiden suckers are taller suckers with wide leaves that have passed the 'sword' leaved stage (Morton, 1987). Water suckers are the suckers from aged and deteriorating corms with wide leaves next to but apparently joined to the mother rhizome. They yield inferior fruits and they are not recommended as propagating material (Crane and Balerdi, 1998). A part of the mother rhizome with a lateral bud is known as corm and it can used for plant regeneration while bits of large corms are called 'bull-head' in Jamaica (Nakasone and Paull, 1998).

The preferred planting materials for cultivating are different in different parts of the world. The rate of growth was found to be affected by the kind of chosen planting materials but they had no effect on bunch size (Nakasone and Paull, 1998). Big sword suckers and maiden suckers are usually planted in Florida. When sword suckers are 1.2 - 1.5 m tall, they are removed from the hardy clumps. The big leaves of the sword suckers are removed and left only the youngest leaves or none at all. The sword suckers normally have many healthy roots and they are checked for diseases and insect infection before used for propagation (Crane and Balerdi, 1998).

2.2 *In Vitro* Culture Technology

2.2.1 Micropropagation of Banana

Micropropagation is an *in vitro* technique of growing plant tissue, cell, or organs isolated from the mother plant on artificial medium (George, 2008). There are several advantages to propagate bananas by using micropropagation technique over traditional methods. Firstly, micropropagation of banana began with small pieces of plant parts such as shoot tips. Hence, maintenance and multiplication of large number of *in vitro* plantlets only need little space. Secondly, aseptic tissues are used in micropropagation and propagation is carried out in aseptic condition to produce disease free plantlets. Thirdly, optimization of culture medium nutrient and plant growth regulator, light and temperature can promote rate of propagation. Forth, micropropagation can be used to produce more and uniform plantlets for banana cultivars that are difficult and slow to propagate asexually. Fifth, production of banana plantlets via *in vitro* techniques will not be affected by seasonal changes and can continue through the whole year (George and Sherrington, 1984).

A wide range of *Musa* genotypes had been established via shoot tip culture by several researchers such as banana 'Dwarf Cavendish' cultivar, Enset (Ensete superbum (Roxb.) Cheesman) and banana cv. 'Basrai' (Mathew and Philip, 1996; Nandwani *et al.*, 2000; Gubbuk and Pekmezci, 2004). Micropropagation of bananas commonly divided into several stages: mother plant selection, initiation, multiplication, rooting and acclimatization. Mother plant selection (stage 0) is extremely important and needed to prevent multiplication of undesirable banana genotypes (Matsumoto and Silva Neto, 2003). When a mother plant was selected, the characteristics of true-to-type, fruit characteristic, growth rate, the health and appearance of sucker were considered (Israeli *et al.*, 1995). Since commercial

explant materials are not checked routinely with virus indexing, mother plant should be chosen based on historic performance records that are virus disease free, growing vigorously and have at least three cycles of banana production. While suckers should be picked from flowering plants to ensure the quality and true-to-type of the mother plant (Israeli *et al.*, 1995; Matsumoto and Silva Neto, 2003).

After stock plant selection, shoot tips were used as explants for culture initiation (stage 1) (Vuylsteke, 1998). Small sword suckers were normally chosen due to easiness of handling without damaging the mother plant during their removal. Suckers collected from the field were washed and trimmed by discarding the most outer tissues of the banana corm and leaf sheaths before transferred to the laboratory (Teisson and Cote, 1997). The corm tissue, leaf bases and outer leaves of chosen explants were cut to 2.5 x 2.5 x 5.0 cm and surface-sterilized with sodium hypochlorite added with a surfactant. Then the shoot tips (5.0 x 5.0 x 5.0 mm) were excised under aseptic conditions after rinsing with sterile distilled water (three times) and inoculated onto the culture medium directly (Israeli *et al.*, 1995). Several sterilization methods were compared for obtaining aseptic initial explant tissues of banana (Hamill *et al.*, 1993).

The size of the primary explant is a crucial factor for effective culture establishment (Israeli *et al.*, 1995). Smaller explants increase the chance of obtaining virus-free plants but high mortality rate and grow very slowly (Matsumoto and Silva Neto, 2003). If the goal of culture establishment was to produce virus-free plants, minimal size of explants was necessary. Conversely, larger explants were preferred for the purpose of rapid multiplication and explants of 5 mm considered to be a suitable size for effective propagation process (Israeli *et al.*, 1995). Explants of larger size (5 - 10 mm) did not enhance the rate of culture establishment instead resulted in

more losses caused by microbial contamination (Josekutty *et al.*, 2003). While, explants more than 10 mm resulted more phenolic browning and contamination (Matsumoto and Silva Neto, 2003).

The most widely used culture medium was Murashige and Skoog (MS) (1962) mineral salts and added with cytokinin [2 - 5 mg/L 6-benzylaminopurine (BA)] to induce bud proliferation (Teisson and Cote, 1997). Culture commonly took place on solid medium with 16 hours light cycle of 1000 - 3000 Lux fluorescent light and at temperature of 28 ± 2ºC. The explants on the culture medium turned green and swelled after a few days. Subculture normally was performed after 4 - 6 weeks or earlier if browning, caused by phenolic oxidation occurred. Browning could be reduced by reducing lighting time and temperature of the culture room (Israeli *et al.*, 1995). Activated charcoal (Nandwani *et al.*, 2000) or ascorbic acid (Banerjee and Langhe, 1985; Gupta, 1986; Ko *et al.*, 2009) could be added into the initiation medium to reduce browning. The most preferred method to control browning was frequent subculturing (every 14 days) of the explants to fresh medium during the first two months of first time inoculation (Banerjee and Langhe, 1985; Hamill *et al.*, 1993; Vuylsteke, 1998).

Formation clumps of small corms or clusters of small shoots normally appeared after 12 - 18 days of culture (Matsumoto and Silva Neto, 2003). Multiplication (stage II) was carried out by subdividing the bud clusters or newly formed shoots and subculturing them on a fresh medium. The multiplication medium was normally very similar to the initiation medium at stage I (Teisson and Cote, 1997). The most common cytokinin being used is BA for shoots induction and multiplication. The multiplication rate was found to be a direct function of the concentration of BA (Vuylsteke, 1998). However, BA of more than 10 mg/L added

into the culture medium might decrease shoot multiplication. Shoot multiplication rate was varying with different amount of cytokinin for various banana cultivars (Wong, 1986). More multiple shoots could be obtained by subculturing the shoots in shoot multiplication medium every 4 - 6 weeks. Larger shoots were cut longitudinally and each half was subcultured as individual (Israeli *et al.*, 1995). The multiplication rate depends on genotype, concentration of cytokinin, size of initial explant, photoperiod and age of culture (Vuylsteke, 1998). When the demand for the production of *in vitro* banana plantlets was low, the multiplication process could be slowed down by changing the culture conditions through lowering the temperature to 15 - 16ºC and reducing the light intensity in culture room. The *in vitro* banana plantlets might be kept in culture without subculturing for more than one year under these culture conditions (Banerjee and Langhe, 1985; Van den houwe *et al.*, 1995).

After the multiplication stage, shoots had to be elongated and rooted in rooting stage (stage III) before being hardened to cope with the external environment. This step was imperative because the bud and shoot sizes multiplied during multiplication stage were very small and not able to remain alive in soil (Vuylsteke, 1998). Roots could be induced on the same medium used for multiplication but sometimes root formation was inhibited by the presence of high cytokinin level during multiplication stage (Teisson and Cote, 1997; Matsumoto and Silva Neto, 2003). Therefore, most of the researchers used rooting medium without cytokinin (Vuylsteke, 1998) but some researchers observed that the use of cytokinin promoted rooting (Gupta, 1986; Wong, 1986) and elongation of plantlets (Farahani *et al.*, 2008). Quite often IBA (Mathew and Philip, 1996; Nandwani *et al.*, 2000) or addition of NAA into the culture medium were used (Gupta, 1986; Matsumoto and Silva Neto, 2003; Kalimuthu *et al.*, 2007). Sometimes activated charcoal was added

in rooting medium for rooting purpose (Gubbuk and Pekmezci, 2004) but was claimed to be non-essential (Vuylsteke, 1998).

If at multiplication stage, the leafy shoots were at least 1 cm long, white cord roots normally produced in 4 - 14 days after transferred to rooting medium. Shoot elongation was necessary to achieve rooting if the explants were bud-like structures (Vuylsteke, 1998). Full strength MS medium could induce sufficient rooting but it was a common procedure to induce rooting on half strength MS medium, for instance, *Musa textilis* Nee (Mante and Tepper, 1983) and *Musa* spp. (Matsumoto and Silva Neto, 2003). In addition, Mante and Tepper (1983) even decreased the content of sucrose to 1 - 1.5% (v/v). At the end of the rooting stage, approximately 4 - 5 cm tall plantlets with four or five leaves (well-established lamina), 4 - 5 mm diameter base of pseudostem and a well-growth root system were ready to be acclimatized (Israeli *et al.*, 1995).

Acclimatization stage (stage lV) is a transition stage where plantlets have to be adapted to temperatures changes, increase in light intensity, lower nutrient availability and decrease in humidity (Matsumoto and Silva Neto, 2003). *In vitro* plants have minimal stomata activity, sparse mesophyll development, numerous intracellular cavities and smaller amount of developed cuticle than *ex vitro* plants. Therefore step-by-step hardening to *ex vitro* condition is crucial for banana plantlets at this stage because they were going through physiological and anatomical changes. After the elongation and rooting stage, plantlets were removed from the culture vessels and agar was gently removed from the roots to prevent the development of pathogens (Israeli *et al.*, 1995). Thereafter, cluster of plantlets had to be separated into individual plants by trimming through the basal corm tissue and then the

individual plantlets were transferred to the potting mixture (topsoil or a composted soil mixture) without damaging the fragile roots (Vuylsteke, 1998).

Normally new roots emerge 4 - 5 days and new leaves begin to grow in less than 8 - 10 days after the starting of acclimatization. The first batch of roots branch from the original roots and subsequent new roots establish from the corm. The control of water supply and irradiation are the most vital factors at this stage (Israeli *et al.*, 1995). Hence the potting mixture needed to be kept moist at the beginning and plantlets were watered directly after transplanting. Furthermore high humidity should be maintained and could be accomplished by frequent misting or putting the plantlets under a humidity chamber. Plantlets were kept partly shaded initially and gradually hardened by progressively reducing the shade and humidity. Diluted solution of fertilizer was applied 4 - 6 weeks after transplantation to encourage the plantlets growth (Vuylsteke, 1998).

Matsumoto and Silva Neto (2003) used bench-type boxes filled with substrate mixture (substrate: vermicultie 1:1) and sheltered with a plastic sheet for 7 - 10 days. After the plastic sheet was removed, the plantlets were grown under frequent mist until they were 20 cm high and transplanted into black polyethylene bags containing 1500 - 2000 cm^3 sterilized top soil. They were grown in a greenhouse (50% shade), watered by a sprinkler system and were given fertilization weekly. After the plants acclimatized in the greenhouse for 10 - 12 weeks, they were ready for transplanting to the field. Field planting preparation and crop management should be carried out to attain successful field establishment (Israeli *et al.*, 1995).

2.2.2 Limitations of Banana Micropropagation

The major limitation of practicing banana micropropagation is somaclonal variation. It is a genetic variation occurs during clonal proliferation of banana (Matsumoto and Silva Neto, 2003). Various morphological changes (off-types) in somaclonal variation were dwarfism, mosaic type, gigantism, foliage colour abnormality and pseudostem abnormality (Israeli *et al.*, 1995). The somaclonal variation rate of *Musa* plants vary from 0 to 69.1% and the acceptable rate for commercial micropropagation is 3 - 5% (Sahijram *et al.*, 2003). Bairu *et al.* (2006) claimed that the multiplication rate and number of subcultures were correlated with the somaclonal variation rate in Cavendish banana. Therefore the number of subculture should be limited. Early detection and elimination of off-types during acclimatization could reduce the loss and limitation of banana micropropagation (Mendes *et al.*, 1999).

Another limitation is high investment in practicing the micropropagation techniques due to the requirement of special infrastructure in laboratory and nursery (Israeli *et al.*, 1995). In addition, high production cost of micropropagation using the conventional gelled culture mainly was due to labor cost (40 - 60%) that needed for periodic subculture (every 4 - 6 weeks) by cutting and planting explants on solid medium manually (Chu, 1995). Besides, the requirement of agar products and numerous small containers in solid culture increase the production cost and complicated automation. High production costs normally limit the commercial micropropagation of high market price plants such as foliage plants, ornamentals and selected fruit crops (Simonton *et al.*, 1991).

2.2.3 Advantages and Limitations of Liquid Culture System

Micropropagation using liquid medium is suitable for reducing the production costs through mass production of plants by scale up and automation techniques. The use of liquid medium in banana micropropagation has many advantages over solid culture system: (1) cleaning of containers was easier and media could be easily changed without transferring the explants. (2) larger container volume could be used and applied with uniform culturing conditions. (3) reduce manual labor and transfer time in subculture by just putting the explants into the liquid medium (Berthouly and Etienne, 2005). (4) nutrient uptake was available for the whole explants and sterilization was feasible by microfiltration. (5) gelled agents were not required and thus reduced the production cost (Robert *et al.*, 2006).

In addition, plants from various species cultured in liquid medium have responded better than those cultured in solid medium. For example, a larger increase in number and length of shoots and also number of roots were observed in turmeric (*Curcuma longa* L.) (Salvi *et al.*, 2002). Generally with *Isoplexis canariensis*, multiple shoot formation was greater and the length of shoots was double in liquid medium than solid medium (Arrebola *et al.*, 1997) and more shoots with greater fresh weight of *Populus alba* x *P. freandidentata* were produced in liquid mediun than agar medium (Chun *et al.*, 1986). Liquid medium provided close contact between nutrient and phytohormones with the whole explants which enhancing root and shoot growth (Ziv, 1989; Smith and Spomer, 1995; Sandal *et al.*, 2001).

Nevertheless, the use of liquid medium in micropropagation also has limitations such as hyperhydricity, asphyxia and shear forces (Berthouly and Etienne, 2005). Several methodologies had been developed to prevent these problems, for instance, culture supports such as polyurethane foam (Conner and Meredith, 1984)

and floating membrane raft (Watad *et al.*, 1995; Watad *et al.*, 1997), liquid medium was added to established culture on agar medium (Maene and Debergh, 1985; Ziv and Shemesh, 1996) and mist culture (Weathers and Giles, 1988; Towler *et al.*, 2006). Temporary immersion system (TIS) could also be used to eliminate hyperhydricity and asphyxia in micropropagation by adjusting immersion time (Berthouly and Etienne, 2005).

2.2.4 Temporary Immersion System (TIS)

2.2.4.1 Principle and Advantages of TIS

TIS is a system that based on the principle that temporary contact between explants with the liquid medium is better than permanent contact (Berthouly and Etienne, 2005). TIS combined the advantages of both solid and liquid culture system. Solid culture system enabled aeration but full contact between whole explants and nutrient media was not provided while liquid culture system allowed nutrient uptake effectively but hyperhydricity was often occurred (Smith and Spomer, 1995; Berthouly and Etienne, 2005). Therefore the reason for the effectiveness of TIS was due to the ability to aerate explants and provided partial or total contact between entire explants and the liquid medium (Alvard *et al.*, 1993).

An intermittent immersion method that explants were exposing alternately to the air and only immerse in the liquid medium temporarily was first described by Steward *et al.* (1952). A tilting device according to temporary immersion principle was then designed by Harris and Mason (1983) and the first automatic machine based on TIS principle was invented by Tisserat and Vandercook (1985). Wide range of semi-automatic systems based on temporary immersion concept were developed and numerous species were produced successfully in TIS, for instance, somatic

embryos of *Hevea brasiliensis* (Etienne *et al.*, 1997), *Psidium guajava* L. (Kosky *et al.*, 2005) and *Camptotheca acuminata* (Sankar-Thomas *et al.*, 2008) as well as shoots of sugarcane (Lorenzo *et al.*, 1998), pineapple (Escalona *et al.*, 1999) and banana (Roels *et al.*, 2005) or potato microtubers (Jiménez *et al.*, 1999) and yam microtubers (Jova *et al.*, 2005).

The use of TIS in micropropagation has several advantages over conventional methods of micropropagation (solid and agitated liquid culture system): eliminates hyperdydricity phenomenon, enables aeration and sufficient mixing, allows medium changes and automation easier, decrease shear levels and reduces cost production. TIS is very effective for shoot proliferation, somatic embryogenesis and microtuberization (Berthouly and Etienne, 2005).

2.2.4.2 Designs of TIS

TIS has four different designs: rocker or tilting machines systems, complete immersion with liquid medium renewal systems, partial immersion with liquid medium renewal systems and complete immersion through pneumatic transfer of liquid medium without medium restoration systems.

First design of rocker or tilting machines was described by Harris and Mason in 1983. Two machines were used, one for rocking 120 units of 455-mL or 70 units of 910-mL wide-mouth Mason jars and another for tilting 400 units of 50-mL or 320 units of 125-mL Erlenmeyer flasks. This design did not include replenishment of liquid medium (Harris and Mason, 1983). Second design of complete immersion with liquid medium renewal systems was used to culture organs, plant tissues and complete plantlets by intermittently drained and refilled with fresh medium in aseptic circumstances. The automated plant culture system (APCS) composed of two

impeller pumps, silicone tubes, two medium reservoir bottles, a plant culture chamber, 3-way steel valve and an interface module consist of relay boards. A micro-computer in APCS was used to control medium introduction, evacuation and restoration in an aseptic condition (Tisserat and Vandercook, 1985). Third design of partial immersion with liquid medium renewal systems always used culture supports (agar medium, cellulose plugs and propylene screen) to support the plant tissue and only partial base of plant material was immersed. Two models of this design had been created (Berthouly and Etienne, 2005). First model was a semi-automatic process used for optimization of shoots production and rooting of *in vitro Pinus* spp. on the agar medium in the same vessel by addition and removal of liquid medium with the aid of peristaltic pumps periodically and automatically. The liquid was transferred onto the agar medium for four to six hours and then drain-off by using a vacuum suction system (Aitken-Christie and Davies, 1988). Maene and Debergh (1985) reported the positive effect of adding liquid medium to agar medium in stage II cultures. Second model was a system using a computer to control the pump to apply liquid medium to plants periodically in 7-liter vessels. Control capacities involved medium introduction and depth regulation inside four culture vessels, medium cycling on an allocated timetable, timetable adaptation during a culture period and medium renewal. Maintaining aseptic condition was important for the growth of the plants culture in this device (Simonton *et al.*, 1991).

The modified TIS design used for mass production of Pisang Awak plantlets in this study was similar to the design of complete immersion through pneumatic transfer of liquid medium without medium restoration systems. This TIS design allowed full contact between whole explants with liquid medium by force ventilation that push liquid medium towards explants and enabled complete renewal of aeration.

Explants could be placed to the container without positioned on a support and liquid was transferred from a tank to the explant container. Over-pressure was provided by an air compressor connected to a timer to control the frequency and duration of medium immersion in TIS. The culture medium of this system has to be replaced every 4 - 6 weeks as fresh medium tank was not included. Currently the design of Automated Temporary Immersion system (RITA®) and Twin Flask system (BIT®) were widely used in the market (Berthouly and Etienne, 2005).

RITA® system consists of two compartments. The upper compartment contains explants and the lower compartment contains liquid medium. Over-pressure from air compressor was applied to lower compartment and push the liquid medium into the upper compartment. The explants were immersed with liquid medium and aerated as long as over-pressure was applied. The liquid medium flowed back into the lower compartment by gravity when the pressure was not supplied. The return of liquid medium to the lower compartment could become faster by using solenoid valve in the air circuit (Alvard *et al.*, 1993). RITA® system usually was used to mass propagate somatic embryos of various plant species (Berthouly and Etienne, 2005). RITA® system was also proven to be an effective propagation system. Sixteen *Crescentia cujete* shoots were immersed into liquid medium for three minutes every three hours in a RITA® temporary immersion system and found to induce a significant increase in leaf number, shoot height, biomass and enhanced transplanting efficiency (Murch *et al.*, 2004). According to McAlister *et al.* (2005), RITA® induced four to six-fold increase in axillary bud proliferation of six *Eucalyptus* clones as compared to semi-solid media. Kosky *et al.* (2005) reported that RITA® system induced significant higher germination percentage and fresh weight of somatic embryo of *Psidium guajava* L. if compared with those attained from

semisolid culture medium. Several other researchers also had reported better results in yield using the RITA® system in comparison with the conventional micropropagation methods. For example, banana (Alvard *et al.*, 1993), *Hevea brasiliensis* (Müll. Arg.) (Etienne *et al.*, 1997), *Coffea arabica* (Barry-Etienne *et al.*, 1999), potato (Teisson and Alvard, 1999), tea clone 'TRI-2025' (Akula *et al.*, 2000), apple root stock 'M9 EMLA' (Chakrabarty *et al.*, 2007), *Hydrastis canadensis* L. (He *et al.*, 2007) and sugarcane (Mordocco *et al.*, 2009).

BIT® system composed of two containers connected by glass and silicone tubes. Explants were inoculated into one container while the other container held the culture medium. Air was supplied via 0.2 μm hydrophobic filters into the culture container with the aid of an air compressor. Pressure created by the air enabled the culture medium to flow back and forth into the culture vessels. The duration of immersion was controlled by an electronic timer and on/off operation was controlled by three-way solenoid valves (Escalona *et al.*, 1999). This system was suitable for organogenesis of plant species (Berthouly and Etienne, 2005). The multiplication rate of sugarcane shoots in BIT® system was doubled in comparison with the solid and liquid culture medium and 46% of significant cost reduction was achieved (Lorenzo *et al.*, 1998). High shoot multiplication was obtained in BIT® system when *Phalaenopsis* shoots immersed with liquid medium for 10 minutes with eight immersions per day after 12 weeks (Hempfling and Preil, 2005). According to Roels *et al.* (2005), plantain in BIT® system had significantly higher multiplication rate than semi-solid medium. Niemenak *et al.* (2008) reported that the number of embryos produced was significantly higher in BIT® system if compared to solid medium after three months of culture. Several other authors also had reported better results in plantlet production using the BIT® system than the conventional

micropropagation systems, for instance, potato (Akita and Takayama, 1994), pineapple (Escalona *et al.*, 1999), *Hippeastrum* x *chmielii* Chm. (Ilczuk *et al.*, 2005), yam (Jova *et al.*, 2005) and *Cymbopogon citratus* (Quiala *et al.*, 2006).

2.2.4.3 Uses of TIS in Plant *In Vitro* Culture Technology

Temporary immersion system enhances shoot proliferation and growth of plantlets. Proliferation of banana cv. Grande Naine shoots via TIS was found to be better than four other liquid medium systems and gelled medium (Alvard *et al.*, 1993). Roels *et al.* (2005) also reported that multiple shoot formation of plantain in TIS was significantly higher than those obtained from semi-solid medium.

Serviceberry shoots (*Amelanchier* x *grandiflora* Rehd. 'Princess Diana') were cultured in intermittent cycling liquid medium of seven-liter vessel (TIS) produced higher proliferation than other four tested methods (Krueger *et al.*, 1991). According to He *et al.* (2007), multiple shoot formation of goldenseal (*Hydrastis canadensis* L.) in TIS was significant higher than those obtained from paper-support liquid, liquid flask and solid cultures. Multiplication of microcuttings in *Coffea arabica* and *C. canephora* using TIS within six weeks was similar with those achieved from solid medium cultured for three months (Berthouly *et al.*, 1995).

Pineapple (*Ananas comosus*) cv. Smooth Cayenne could be produced over 3000-fold of multiple shoots within six months using TIS (Firoozabady and Gutterson, 2003). Besides, Quiala *et al.* (2006) demonstrated that TIS produced higher shoot multiplication with increased fresh weight and dry weight of *Cymbopogon citrates* compared with those from semi-solid culture. Micropropagation of banana terra using TIS induced 2.2 times more shoots and 2.86 times more biomass than the semi-solid culture (Lemos *et al.*, 2001). According to

Murch *et al.* (2004), TIS significantly increased the growth of *Crescentia cujete* plantlets. Biomass, shoot height and leaf number of the plantlets grown in TIS was significantly higher than those obtained from liquid and solid culture.

TIS was used to mass produce coffee (*Coffea arabica*) callus. The mass production of several tested callus genotypes in TIS was found to be better than those attained in Erlenmeyer flasks. This could be due to callus was being immersed continuously in Erlenmeyer flasks and in contact for a long period with toxic substances (phenols) released in the liquid medium. Therefore, the use of TIS to mass produce callus could solve this problem as immersion of one minute per day in TIS only allow intermittent contact between callus and toxic substances in liquid medium (Berthouly *et al.*, 1995).

Physiological effect of TIS on *Hevea brasiliensis* callus was evaluated by Martre *et al.* (2001). Different parameters of metabolic activity of friable embryogenic callus were investigated by using TIS while the conventional methods of agitated liquid and semi-solid cultures were used as controls. The lipid peroxidation and superoxide dismutase activity in TIS using 12 and 24 hours immersion treatments were significantly higher than the controls.

According to Akita and Takayama (1994), tuberization and plant growth of potato (*Solanum tuberosum* L.) were evidently stimulated by twin flask system of TIS. TIS induced about 500 - 960 tubers in a 10-liter jar fermentor after ten weeks. In addition, the total mass of tubers produced was also increased in TIS. On the other hand, there was no tuber formation in complete and continuously immersion system. Furthermore, Teisson and Alvard (1999) used double RITA® of TIS to produce potato microtubers for three different cultivars (Bintje, Ostara and Desiree). An