MICROPROPAGATION OF PINEAPPLE (*ANANAS COMOSUS*) CVS. SMOOTH CAYENNE AND MORIS

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Plant Tissue Culture

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Abstrak

Sejumlah 25 eksperimen telah dijalankan untuk mengkaji kesan beberapa faktor seperti jenis-jenis hormon dan kepekatan, masa inkubasi, subkultur, keadaan medium, sukrosa, pH, saiz eksplan, ketumpatan eksplan dan kandungan medium untuk setiap kultur dan kekuatan medium terhadap pertumbuhan pucuk untuk setiap eksplan, jumlah pucuk, jumlah kos dan kos untuk setiap pucuk. Setiap kultivar Smooth cayenne dan Moris disubkultur selama 4 kali berturut-turut . Respons terhadap akar dan kualiti plantlet terhadap kedua-dua kultivar telah dikaji di dalam hormon yang berbeza, kekuatan media, umur pucuk, kandungan sukrosa dan masa inkubasi. Penilaian kebolehidupan semasa aklimatisasi berkaitan dengan perlakuan akar, kualiti plantlet, jenis substrat dan nisbah percampuran dibawah polyethylene telah ditentukan. Dua protokol yang mudah dan berkos rendah telah dirangka untuk propagasi secara komersial terhadap kultivar Smooth cayenne dan Moris. Prosedur ini melibatkan kultur aseptik eksplan hujung pucuk yang diambil daripada ‘crown’ kultivar Smooth cayenne dan pucuk daripada kultivar Moris yang ditanam secara konvensional diikuti oleh penggandaan pucuk, akar dan aklimatisasi. Sebanyak 121,125 bahan tanaman Smooth cayenne boleh dihasilkan setiap tahun daripada satu pucuk yang berharga 26 sen untuk setiap propagul. Satu pucuk untuk setiap kultur telah melalui 4 kali kitaran penggandaan pada setiap 75 hari di atas 20 ml agar pada kekuatan penuh MS yang ditambah dengan sukrosa sebanyak 30 g/l, kombinasi BAP pada 3.25 mg/l dan IAA pada 1.75 mg/l dan pH 5.7. Jumlah pucuk , jumlah kos dan kos untuk setiap pucuk pertama dan selepas 1, 2, 3 dan 4 subkultur ialah 17; 323; 8075 dan 12 1125 pucuk, RM 1.61; RM 28.63; RM 542.82 dan RM 13,437.36 dan 6.5 sen, 6.2 sen, 4.74 sen dan 7.8 sen. Pada masa yang sama, jumlah 15,120 bahan yang di tanam bagi nenas kultivar Moris setiap tahun mungkin boleh dihasilkan bermula 12 pucuk pada harga 23 sen untuk setiap propagul. Pucuk-pucuk telah dikultur pada ketumpatan 4 pucuk setiap kultur dan

melalui 4 kali kitaran penggandaan setiap 60 hari di atas 8 ml cecair MS berkepekatan penuh MS yang ditambah dengan sukrosa sebanyak 20 g/l, 2.0 mg/l BAP dan pH 5.0. Jumlah pucuk yang pertama dan selepas 1, 2, 3 dan 4 subkultur adalah 84, 504, 3024 dan 15120 pucuk, dan berharga RM 2.98, RM 21.96,RM 134.26 dan RM 806.51 manakala kos untuk setiap pucuk 4 sen; 4 sen; 4 sen dan 5 sen.

Pucuk bagi kedua-dua kultivar telah mengeluarkan akar pada ketumpatan 5 pucuk setiap kultur dalam 6ml cecair pada kekuatan ¼ MS yang ditambah dengan sukrosa pada 20 g/l, tetapi hormon yang berbeza. 1.0 mg/l NAA telah digunakan untuk menumbuhkan akar kultivar Smooth cayenne dan IBA 2.0 mg/l untuk menumbuhkan akar kultivar Moris, pH 5.0 dan kultur diinkubasi untuk 30 hari. Plantlet kedua-dua kultivar telah diaklimatisasi (>84%) di dalam pasu yang dipenuhi dengan pasir dan disertai dengan kepingan polyethylene selama 15 hari dan disimpan di bawah rumah hijau selama 75 hari. Kos untuk menumbuhkan akar tersebut dianggarkan sebanyak 13 sen setiap pucuk dan aklimatisasi adalah 5 sen setiap plantlet. Kapasiti penghasilan boleh ditingkatkan kepada mana-mana peringkat, dengan mengutamakan bahan permulaan eksplan.

Abstract

A total of 25 experiments were conducted to test the effect of several factors such as hormone types and concentrations, incubation periods, subcultures, medium states, sucrose, pH, explants size, explants density and medium volume per culture and medium strength on the shoot formation per explant, total shoots, total cost and cost per shoot of Smooth cayenne and Moris pineapple at each and over 4 consecutive subcultures. Rooting responses and plantlets quality of both cultivars were tested in different hormones, media strength, shoots age, sucrose content and incubation periods. Survival during acclimatization was evaluated in relation to rooting treatments, plantlet quality, substrate types and mixing ratio under polyethylene enclosure. Two protocols of low cost and simplicity were developed for commercial propagation of Smooth cayenne and Moris pineapple. The procedure involved aseptic culture of shoot tips explants excised from crown of Smooth cayenne and shoots of field grown Moris plants followed by shoot multiplication, rooting and acclimatization. A total of 121,125 planting materials of Smooth cayenne could be produced per year from single shoot at cost of 26 cents per propagule. Single shoot per culture underwent 4 multiplication cycles each 75 days long on 20 ml of agar solidified full strength MS enriched with sucrose at 30 g/l, combination of BAP at 3.25 and IAA at 1.75 mg/l and pH adjusted to 5.7. The total shoots, total cost and cost per shoot at the first and after 2, 3 and 4 subcultures were 17; 323; 8,075 and 12,1125 shoots, RM 1.61; RM 28.63; RM 542.82 and RM 13,437.36 and 6.5; 6.2; 4.7 and 7.8 cents respectively. Similarly, a total of 15,120 planting materials of Moris per year could be produced starting with 12 shoots at cost of 23 cents per propagule. Shoots were cultured at density of 4 shoots per culture and underwent 4 multiplication cycles each 60 days long on 8 ml of liquid full strength MS enriched with sucrose at 20 g/l, BAP at 2.0 mg/l and pH

adjusted to 5.0. The total shoots, total cost and cost per shoot at the first and after 2, 3 and 4 subcultures were 84; 504; 3,024 and 15,120 shoots, RM 2.98; RM 21.96; RM 134.26 and RM 806.51 and 4; 4; 4 and 5 cents respectively.

The shoots of both cultivars were rooted at density of 5 shoots per culture in 6 ml of quarter strength liquid MS enriched with sucrose at 20 g/l, but different hormones. NAA at 1.0 mg/l was used for rooting of Smooth cayenne and IBA at 2.0 mg/l for rooting of Moris, pH adjusted to 5.0 and the cultures incubated for 30 days. The plantlets of both cultivars were acclimatized (> 84 %) in pots filled with sands, enclosed with polyethylene sheet for 15 days and kept under shade house for 75 days. The cost of rooting was estimated as 13 cents per shoot and that of acclimatization was 5 cents per plantlet. The production capacity can be increased to any level, preferably by increasing the starting explants material.

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Table of contents

List of Figures

List of tables

List of plates

List of Abbreviations

AFP (% of air filled porosity)

ANOVA (Analysis of variance)

BAP (6-benzylaminopurine).

B5 (Gamborg medium)

Casein hydrolysate (N-Z Amine A from Bovine milk)

Coconut milk (liquid from coconut fruit)

Ctube (Culture tubes)

2,4-D (2,4-dichlorophenoxyacetic acid)

Dicomba (3,6 Dichloro-2-methoxybenzoic acid)

EAW (Easily available water)

GA_3 (Gibberellic acid)

g/l (gram per liter)

IAA (indole-3-acetic acid)

IBA (indole-3-butyric acid)

2iP (6-(γ, γ-dimethylallylamino) purine)

KN (Kinetin) 6-furfurylaminopurine

Mdis (Medium dispensing)

mg/l (milligram per liter)

Mprep (Medium preparation)

MS (Murashige and Skoog medium)

MT (Murashige and Tucker medium)

N (Nitch basal medium)

NAA (naphthaleneacetic acid)

RM (Ringgit Malaysian; currency)

Picloram (4- Amino-3,5,6-trichloro-2-pyridinecarboxylic acid)

SH (Schenk and Hildebrandt medium)

White (White's medium)

WPM (Woody plant medium)

ZN (Zeatin) (6-(4-hydroxy-3-methyl-but-2-enylaminopurine)

Introduction

Pineapple is herbaceous, perennial plant belongs to Bromeliads family, scientifically named *Ananas comosus* L. (Merr.) and economically classified into five groups; Cayenne, Queen, Spanish, Abacaxi and Maipure. The first two groups are the most important ones as 90 % of world production (18.2 million tons) comes from varieties belong to these two groups. Pineapple plants are characterized by short stems (30 cm) with very short condensed internodes. The stem is completely hidden by about 80 narrow, sessile, fibrous, 90 cm long leaves arranged around the stem in 5/13 phyllotaxy giving the plant a rosette shape. Each leaf base covers the other; the oldest is outside and the youngest inside up to the center where the terminal bud is located. The plant is short with about 75 to 150 cm height and 90 to 120 cm spread and the root system is shallow fibrous about 30 cm deep.

Pineapple has one terminal bud at the top of the stem and lateral bud at the base of each leaf. Both of the vegetative and reproductive growths of the pineapple originate from the terminal bud. When the terminal bud changes to reproductive mode, it gives an inflorescence that would develop into parthenocarpic fruit. After fruit set, the terminal bud reverts back to vegetative mode giving a branch at the top of the fruit called crown. During fruit maturation, some of the buds located at the leaves base sprout giving various types of branches known by different names according to their location on main stem (Suckers and Shoots) and fruit peduncle (Slips and Hapas). When the fruits ripe within 18 months from planting, the mother plant completed the growth cycle and collapse. The suckers (branches arise from stem part below the ground) take over giving new pineapple plant for the second cycle of growth and fruit production. For convenient, conduction of cultural practices the pineapple fields are usually re-established every 3 to 6 years (Bartholomew *et al.*, 2003).

Introducing and developing of superior varieties with economical horticulture traits through conventional breeding and genetic engineering are the most effective means for solving low yield, poor quality and physiological disorder problem facing pineapple farmers. However, the main obstacle would be shortage of planting materials. Pineapple is usually planted at high density (25 000 up to 80000/ hectare) while one plant produces only three propagating materials (one sucker, two slips per two years). Following conventional methods of propagation such as segmentation of vegetative parts and single leaf unit could not solve the propagules shortage. Tissue culture has the potential of rapid mass production of propagules. Previous work showed that several tissue culture protocols using solid, stationary and shaking liquid and temporary immersion systems have been proposed for pineapple propagation. Close look into these protocols indicated that *in vitro* induction of shoot multiplication, rooting and potential for mass production is well documented but optimization of establishment of primary explants, multiplication and rooting stages is very far from being reached and commercial production of propagules using any of these reported protocols is questionable. Many of the factors that could affect multiplication and rooting were not investigated and great deal of crucial information concerning the cost items and the effect of various factors of multiplication, rooting and acclimatization on the total cost of production as well as cost per propagule were totally ignored.

Several researchers (Be and Deburgh, 2006, Firoozabady and Gutterson, 2003, Soneji *et al.,* 2002a) recommended different treatments as the best treatment for multiplication and rooting of pineapple. Yet, all of these factors except hormone and medium states received very little attention. For instance, medium types and strength, medium volume dispensed per culture, sucrose content, explants size and density, shoot age (number of subcultures) and incubation periods which not only affect multiplication rate and *in vitro*

rooting but also have direct effect on cost of both multiplication and rooting were arbitrary picked up, kept at one fixed level and tested at one fixed incubation period. None of these factors were tested alone at different levels or in combination with others factors**.** In fact, in some studies not only some of these factors were not tested but also were not even reported. In many cases, it was very difficult to figure out what was the size of the shoots used for multiplication and what happen to the other shoots of different sizes. Whether the shoots of different sizes have equal shoot formation capacity was not investigated and the volume of medium used per culture during multiplication and rooting stages was not mentioned.

Shoot formation rate per explant is very important parameter for assessment of shoots multiplication treatments, but in almost all studies the rate was reported as average of several subcultures rather than at each subculture. These reported shoot formation rates overall subcultures did indicate potential for mass production but not enough for assessment of feasible commercial production. The rate per explant and the total shoots have to be presented at each and over consecutive subcultures per unit of medium (one liter) rather than the overall average of shoot formation per explant. Total shoots per liter is a very practical parameter for selection of treatment with higher multiplication at lower cost. Furthermore, in several studies, an expected total shoots after several months and even year was estimated but this total shoots expectation was just an extrapolation extended to period longer than the actual experimentation time. Not only both data could not be considered valid for assessment of different treatments but also both the overall rate and yearly expectation of production could not be used for proper management of micropropagation and evaluation of the cost.

Most of the focus was on multiplication and little attention has been given to the *in vitro* rooting of pineapple. The suggested rooting treatments in over 50 % of the proposed

protocols for pineapple micopropagation were just mere statement with no supporting data and the cost of rooting stage was never discussed. Rooting is an important stage conducted mainly to improve the survival during the hardening period and growth during the subsequent acclimatization stage. However, in all rooting experiments the relation between rooting treatments, rooting parameters, plantlets quality and acclimatization was not investigated. If the rooting remained conducted the way it is usually reported, complaint about the high cost of micropropagated materials would always be there even if the multiplication was fully automated and at highest rate.

Commercial pineapple micropropagation was vaguely claimed without providing supporting details of cost analysis. It is very difficult to comprehend commercial production while the cost of the factors that affect the multiplication rate and rooting not only were not itemized but also were not even taken into consideration. In many of the proposed micropropagation protocols, the medium volume and explants density per culture were not even mentioned and the multiplication rate per explant, total cost, total shoots and cost per shoot at each subculture were not reported. Without testing the effect of different type and strength and pH adjustments of media, density, size and age of the explants and medium volume dispensed per culture, treatment with highest possible multiplication and lowest cost could not be achieved. Without knowing the changes in shoot formation rate and cost over subcultures neither the feasibility nor proper plan and management of the project could be layout. Optimization of pineapple *in vitro* multiplication and rooting and commercial production of pineapple propagules have not been reached yet. More factors and different combinations of factors still need to be tested and new methods and parameters need to be introduced for efficient and valid assessment and evaluation of multiplication and rooting treatments.

Moris pineapple is the most commonly grown local cultivar in Malaysia. Up to date there are only two reports of micropropagation of this cultivar (De Silva *et al*., 2008; 2006; Hamad and Taha, 2003). In one report the shoots were regenerated from callus and on the other from shoot tips and in both reports the rooting and acclimatization was not reported. Smooth cayenne is a famous pineapple world wide. The objectives of this study were to

1. Optimize the *in vitro* multiplication and rooting of pineapple cv. Moris and Smooth cayenne.
2. Establish efficient regeneration system for these two cultivars.
3. Provide an estimate cost at each and over consecutive cycles of multiplication and rooting.
4. Develop a cost effective, better managed system for *in vitro* commercial production of pineapple propagules.

Literature reviews

Intact terminal and axillary buds of crowns, suckers and slips are the most commonly used primary explants for micropropagation of pineapple. In all *in vitro* multiplication procedures, the explants severed from the selected mother plants, sterilized and cultured under aseptic condition into sterilized agar solidified or liquid MS medium supplemented with sucrose and proper hormones. After establishment (obtaining of clean and growing primary explants), the shoots passed to multiplication stage followed by rooting and acclimatization.

1.1. Establishment of terminal and axillary buds

Establishment of primary explants is a very essential key step. If only very low percentage of the primary explants could be established and the explants grow into solitary or few shoots after long incubation, long period of time would be needed before the quantity of the *in vitro* obtained shoots become large enough to test the effect of several factors and combinations on the multiplication capacity of the shoots (secondary explants). A long lag period would also protract the time required for production of large number of propagules (Dewald *et al*., 1988). Successful establishment of primary explants depends on successful sterilization of explants, medium, vessels, tools, restricted sanitation procedures and proper balance of minerals and hormones in accordance to cultivar, explants type and purpose.

1.1.1. Sterilization of medium and explants

Medium, forceps, tweezers, vessels and other glassware and tools are usually sterilized in autoclave at 121 ^{0}C and 1.5 kg/cm^{2} for 15 to 25 minutes. However, Teixeira *et al.* (2006) reported that during multiplication of pineapple, chemical sterilization (using Clorox) of vessels and medium was as effective as autoclaving method. Similarly,

Gonzalez-Olmedo *et al.* (2005) found that the growth and elongation of pineapple shoots in medium sterilized by addition of antibacterial Vitrofural (G-1) was as good as the shoots cultured in autoclave-sterilized medium. Chemical and antibacterial methods are advantageous in term of electricity cost. Filter sterilization was also used to avoid decomposition of hormone particularly Zeatin (ZN) and amino acids during autoclaving. These compounds added to autoclaved medium under laminar floor through autoclaved filter.

Sterilization of pineapple explants, on the other hand, is usually started by defoliation of the plant part, washing and removing dirt under running water for half hour. After being defoliated and washed different sterilization procedures were suggested by different researchers. The most common sterilization agents used for sterilization of pineapple explants were Clorox (house bleach with 5.25 % active chloride), Sodium hypochlorite (NaOCl) at 5 to 30% for 5 to 30 minutes and Mercury chloride ($HgCl_2$) at 0.01 to 0.1 % for 0.5 to 10 minutes. However, while some researchers used only one other used two sterilants and in all cases at different concentrations and exposure time. Some started sterilization after defoliation and before excision of the explants in a single sterilization step using one sterilant agent as $HgCl_2$ (Khan *et al*., 2004), Domestos house bleach (Sripaoraya *et al*., 2001a) and Na OCl (Teng, 1997, Wakasa, 1989). Others applied double sterilization steps by repeating the sterilization twice using the same agent on both steps such as Domentos bleach (Sripaoraya *et al*., 2003) while others used different agents for each step as Salvon bleach and $HgCl_2$ (Rahman *et al*., 2001), Bavistin bleach and $HgCl_2$ (Singh and Manual, 2000). Other excised the explants first and then carried the sterilization in single step using one sterilant agent as $HgCl_2$ (Gangopadhyaya *et al*., 2005; Soneji *et al*., 2002a; Bhatia and Ashwath, 2002; Devi *et al*., 1997; Bordoloi and Sarma, 1993; Mathew and Rangan, 1979; Mathew *et al*., 1976), NaOCl (Smith *et al*., 2002; Wakasa, 1989; Drew,

1980) and Clorox (Liu *et al*., 1989). Double sterilization using 70 % ethanol for 1 to 3 minutes followed by either Clorox (Fernandez and Pomilio, 2003; Hirimburegama and Wijesinghe, 1992), NaOCl (Fitchet, 1993, 1990a), CaOCl (Almeida *et al*., 2002) or $HgCl_2$ (Khatun *et al*., 1997) and triple sterilization using NaOCl, $HgCl_2$ and ethanol (Aydieh *et al*., 2000) were suggested for pineapple shoot tip and lateral buds sterilization. Sterilization was also conducted before and after explants excision using one agent as NaOCl (Ko *et al*., 2006) and Clorox (Dewald *et al*., 1988; Zepeda and Sagawa, 1981) and using bleach before and bleach and hot water after excision of laterals (Broomes and McEvan, 1994). In all sterilization procedures, at the end the explants were removed from sterile solution and rinsed several times in sterilized distilled water, trimmed and placed in suitable growing medium under laminar flow cabinet.

Unfortunately, most of the time the sterilization method as well as the results was briefly mentioned with very limited details and in some time the sterilization method was not reported (Be and Debergh, 2006; Omokoio *et al*., 2001; Das and Bhowmick, 1997; Almeida *et al*., 1997; Daquinta *et al*., 1997) and in case when a percentage of success was reported the result was not reproducible. The mother plants even from a single field are grown under different microclimate and ecosystem. Each individual plant in the field could be under different infestation levels or of different types of microbes that the recommended procedure could not eliminate. The problem aggravated further if the plant growth habit is of condenses overlapping nature and when under ground part used as explants source. In different field, the plants are under different cultural practices and managements of pest control. In addition, different laboratories may follow different sanitation procedures and discipline roles. Aydieh *et al.* (2000) claimed 100 % successfully clean growing shoot tips that were subjected after being excised to triple sterilization procedure, NaOCl (1.5 active Cl) for 20 minutes, $HgCl_2$ (0.1 %) for 30 seconds and ethanol for 5 seconds. Singh and

Manual (2000) reported 76 % success of crown laterals excised after the crown was double sterilized using Bavistin (1 %) for 15 minutes and $HgCl_2$ (0.1 %) for10 minutes. Zepeda and Sagawa (1981) reported 70 % clean growing cultures after sterilizing in Clorox before and after excision of the laterals buds. After treating the crown with Benlate for 3 days before explants excision and the defoliated crown with bleach and double sterilization of the excised lateral with bleach and hot water, Broomes and McEvan (1994) reported 80 % clean growing cultures. Fitchet (1993) mentioned that the 99 % contamination of pineapple treated with 0.2 % calcium hypochlorite (CaOCl) could be reduced to satisfactory results if the concentration increased to 2 % and the smaller the size of the explants (2 mm) the higher the percentage of clean cultures. Wakasa (1989) said that most of the laterals and shoot tips of pineapple exposed to Clorox 20 % for 20 minutes were lost due to sterile injuries while at 5 minutes the losses were mainly due to contamination.

1.1.2. Establishment of explants

In addition to obtaining of only low percentage of microbial free primary explants, clean cultures do not guarantee the explants growth. Dormancy and quiescent state of the buds, cultivars, concentrations and types of hormone, medium types and states and explants manipulations all could delay or even block sprouting and developing of the primary explants into strong solitary shoot, cluster of few shoots or callus. Fitchet (1990a**)** noticed that lateral buds from both freshly harvested and dried crown of Queen pineapple sprouted in the establishment medium while no sprouting obtained from lateral of Smooth cayenne pineapple unless the crown was dried for two or more days before excision of laterals. Broomes and McEvan (1994) emphasized hot water treatment and intactness of buds for breaking of pineapple bud dormancy. Buds which were not treated with hot water showed lower percentage of establishment. All of the buds subjected to half way cut in the base failed to be established in solid MS and only 25 % sprouted in liquid while over 80 %

of intact buds sprout in both states. However, Fitchet (1993, 1990a) mentioned that sectioning of dome to 2 mm thick section did not affect sprouting percentage. Hirimburegama and Wijesinghe (1992) noticed that presence of leaves around the meristem tip delayed its sprout.

Full strength MS medium supplemented with sucrose at 30 g/l and adjusted to pH 5.7 is the most commonly used medium for establishment of pineapple primary explants. Only two researchers used MT (Sripaoraya *et al*., 2003; Fitchet, 1993; 1990 a, b). Three suggested two media for establishment, Nitch salt formulation for 10 days and then transfer to MS formultion (Soneji *et al*., 2002a; Mhatre and Rao, 2002a; Mathew *et al*., 1976). Equal establishment in MS and B5 media was reported by Liu *et al*. (1989). On the contrary, Borodoloi and Sarma (1993) reported best establishment in MS and SH media while the explants failed to be established in B5 and White medium. Omokoio *et al*. (2001) suggested half strength MS while Teng (1997) and Das and Bhomick (1997) diluted MS salt components but kept vitamins at full strength. Firoozabady and Gutterson (2003), on the other hand, suggested MS (half salt) and B5 vitamins while Fernando (1986) used MS (full salt) and White vitamins. Sucrose at 20 (Ko *et al*., 2006; Soneji *et al*., 2002a; Mhatre and Rao, 2002a,b; Smith *et al*., 2002), 35 (Kofi and Adachi, 1993), 40 (Almedia *et al*., 1997) and 50 /l (Sripaoraya *et al*., 2003) and adjusting the medium pH to 5.5 (Teng, 1997) and 6.0 (Broomes and McEvan, 1994) were also suggested during establishment of pineapple culture. Although most of the time solid medium was used during establishment, liquid medium seemed to be more suitable. Broomes and McEvan (1994) reported that all of the buds that were cut at base failed to be established in solid MS and only 25 % sprouted in liquid while over 80 % of intact bud sprouted in both states. Although no reasons were given for these modification of MS medium strength, vitamins components, sucrose content and pH and no comparison were reported, it could be assumed that

preliminary experiment were conducted and these modifications produced better establishment than the non modified MS medium. Different researchers used different hormone treatments during establishment of primary explants. Hence, suitability of certain medium and medium modification would be better judged in connection with hormone it contained.

Devi *et al.* (1997) reported that lateral buds of Queen pineapple crown failed to sprout in MS supplemented with NAA applied singly at 0.1 and 1.0 mg/l and in combination with KN at 1.0 and 2.0 mg/l and in MS supplied with IAA singly applied at 0.1 and 1.0 mg/l and eventually died. While 100 % sprouting and growth obtained when the KN was replaced by BAP at equal concentrations. Bhatia and Ashwath (2002) reported 100% establishment of Smooth cayenne laterals in MS enriched with BAP at 1.0 mg/l. However, in medium enriched with BAP at 0.025 and 2.25 mg/l the establishment declined to 68 and to 20 % respectively. Rahman *et al.* (2001) obtained 100 % establishment of meristem tip of Giant Kew pineapple in MS containing combination of BA at 1.0 and NAA at 0.1 mg/l. But, the establishment declined to 24 % when the concentration of BAP and NAA were increased to 2.0 and 1.0 mg/l respectively. None of the explants grew in hormone free MS medium while in medium containing combination of KN, NAA and IBA the establishment varied from 10 % to 95 % depending on the concentration of the hormones. Similarly, Khatun *et al.* (1997) reported that none of Giant Kew grew in hormone free medium and the establishment percentage in MS enriched with triple combination of NAA, IBA plus KN at different concentrations varied from 70 to 100 %. For Giant Kew inclusion of hormone was essential for sprouting, while sprouting of Smooth cayenne and Queen Fitchet (1990 a) obtained in both hormone free and hormone-contained medium. Mathew and Rangan (1979) obtained 76 % establishment in MS containing NAA (1.8 mg/l), IBA (2.0 mg/l) plus KN (2.1 mg/l) while only 55 % of explants grew in MS containing only KN

at 2.1 mg/l. Hirimburegama and Wijesinghe (1992) noticed than the shoot tip of Smooth cayenne could be established in MS enriched with NAA at 0.02 mg/l alone and in medium enriched with combination of BAP at 2.25 mg/l and IAA at 0.18 mg/l. Complete removal of BAP did not effect establishment. However, when the concentration of BAP of the combination lowered to 0.25 mg/l the explants failed to grow. Almieda *et al.* (1997) reported better establishment of Perola in MS enriched with BAP at 2.0 mg/l and IAA at 1.0 m/l while better establishment of Primavera was obtained in medium enriched with BAP at 3 mg/l and IAA at 1.0 mg/l.

The establishment stage starts from placing the primary explants in the establishment medium and terminated by separating of shoots from primary and transferring to fresh and different medium could be as short as 7 to 10 (Soneji et al., 2002a; Hirimburegama and Wijesinghe, 1992), 14 days (Firoozabady and Gutterson, 2003; Khatun *et al*., 1997), 21 days (Fernando, 1986) to as long as 90 days (Almeida *et al*., 1997). The laterals and meristem tip usually grew into solitary and multiple shoots. Hirimburegama and Wijesinghe (1992) noticed that in all shoot tip of crown, the leaf unfolded after 7 days and Khatun *et al.* (1997), on the other hand, reported 100 % sprouting in 15 days in solid medium. Broomes and McEvan (1994) reported that the percentage of sprouting buds over the first, third and fourth weeks was 35, 65 and 80 % in liquid and 18, 53 and 63 % in semi solid medium. Firoozabady and Gutterson (2003) reported development of 2 to 3 shoots per primary within 30 days, while Mathew and Rangan, (1979) reported formation of 6 to 8 shoots per explant in 8 weeks. Assessment of establishment is usually done by comparing of the percentage of sprouting explants, time to sprout, number and average length of shoots and by percentage and size and weight of callus per primary explant. Treatments which result in highest percentage of sprouting primary explants and more shoot formation per explant in shorter incubation period is better than treatments that result

in highest sprouting percentage but only solitary or few shoots formed per explant and after longer incubation. Generally, a one month period is good enough for maximum establishment percentage of primary explants. However, for maximum shoot formation per primary and to secure obtaining of large number of shoot to start the following multiplication stage, the incubation period (establishment stage) should be extended to two and three months.

Optimization of establishment stage is very difficult task. Optimization requires testing of large number of factors and several combinations and using of large number of primary explants. However, most of the primary explants would be lost due to contamination problem and not because of the factors tested for explants establishment. Unless a successful and reproducible procedure of explants sterilization is available, investigation of the factor affecting primary explants establishment is very difficult. Transferring of the clean and growing primary explants after one or several weeks without separating of sprouting shoots to same or to new media for testing the effect of several factors is not really testing of establishment as the explants are already established and does not terminate the establishment stages. However, it tests the proliferation and growth capacity of the established primary explants. On the other hand, if the shoots separated from the primary and transferred to fresh medium the establishment stage ended and multiplication stage started. One of the drawback of pineapple tissue culture application for commercial production of propagules is the too long lag time before enough shoots available for starting production of large number of propagules (Dewald *et al*., 1988). Developing of procedures and treatments that would result in establishment of higher percentage of primary explants and higher shoot formation per primary in shorter period of incubation and staring with large number of primary explants would shorten this lag time. In addition, successful establishment of highest percentage of primary explants would reduce the

number of multiplication cycle required for production of the target total of shoots and it is one of the strategies to avoid occurrence of somaclonal variation (Smith *et al*., 2002). In all reported pineapple cultures, the investigation of establishment of primary explants were cut short and the researchers satisfied with low percentage of establishment and concentrated in the multiplication of the obtained shoots.

1.2. *In vitro* Multiplication of pineapple shoots

Multiplication is the stage in which the *in vitro* shoots (secondary explants) obtained during establishment is induced to multiply into more shoots. The key for successful multiplication is overcoming the apical dominance, inducing and supporting of multiple shoots formation and growth and the multiplication is usually assessed by rate of shoot formation per explant. If the salt formulation and hormone treatment used during establishment of the primary explants resulted in high rate of shoot formation, the medium used for establishment could also be used for multiplication stage too. The important of multiplication stage is its indefinite repeatability. The shoots transferred to fresh medium just before the medium nutrient supply diminished and the shoot formation and growth start to decline. The multiple shoots complex picked up, divided into quarters or clusters of 2 to 3 shoots or separated into individual shoots and subculture either as intact or halved shoot in the same but fresh medium. The process of subculturing repeated several times by transferring the shoots from one multiplication cycle to the next until the rate of shoot formation per explant per subculture start to decline or the intended total of shoot production obtained.

The best multiplication treatment is that which induce the highest shoot formation rate in the shortest incubation period and the rate increased or remained stable over several subcultures. However, this goal could not be achieved unless combinations of set of

several factors affecting the shoot multiplication capacity are investigated. Large volume of work in pineapple bud culture had been reported in several journals and five pineapple symposiums were held so far. Nevertheless, it is not only difficult to find report that investigated *in vitro* multiplication factors other than the effect of hormones and medium states but also very limited data and contradicting results were provided. Several factors ranging from medium types, strength, states, pH and sugar content and medium volume per culture, explants size, density and age of stock cultures (number of subculture) to pineapple varieties and physical surrounding as vessel size, temperature and photoperiod could directly or indirectly via interaction with other factors affect the explants capability to form new shoots and maintain an increasing rate of shoot formation over several consecutive subcultures. However, investigation of most of these factors individually and in combination with other was in generally ignored or received little attention with no detail provided.

1.2.1. Hormones and Natural additives

BAP is the only hormone that always included either singly or in combination with other hormones in the establishment and multiplication media of pineapple. However, researchers disagreed in the optimal concentration and whether the BAP used singly or in combination with one or two types of auxins (NAA, IBA, IAA), other cytokinin (KN, 2iP, ZN) or natural extracts as coconut milk and Casein hydrolysate. BAP singly applied at concentration of 1.0 mg/l resulted in higher shoot formation rate (4 shoots /30 days) than at 0.5, 1.5 mg/l in agitated culture at 120 rpm (Zepeda and Sagawa 1981), higher rate (7 shoots/30 days) than at 0.5 and 2.0 mg/l in solid and agitated culture at 120 rpm (Aydieh *et al*., 2000), higher rate (9 shoots/60 days) than at 0.5 mg/l in static liquid (Be and Debergh, 2006) and higher rate (74 shoots/ 43 days) than at 0.5, 1.5 and 2.0 mg/l in liquid agitated at 70 rpm (Fernando, 1986) . BAP at 1.0 mg/l for 30 days in agar solidified (Sripaoraya *et al*.,

2001b) and for 35 days in gelrite solidified MS medium (Firoozabady and Moy, 2004) was also recommended for multiplication of pineapple. On the contrary, Khan *et al*. (2004) reported that BAP at 0.5 mg/l in agar solidified MS resulted in higher shoot formation (4 shoots/42 days) than at 1.0 and 1.5 mg/l. Almeida *et al*. (2002) reported that after 5 cycle of multiplication (42 days each) BAP at 1.5 mg/l resulted in higher total shoot than BAP at 3.0 mg/l in solid (14 and 4 shoots) and in liquid medium (250 and 146 shoots respectively). BAP at 2.0 mg/l in solid B5 medium resulted in higher shoot formation (7 shoots/ 30 days) than 0.5, 1.0 and 2.5 mg/l (Bhatia and Ashwath, 2002) and in agar solidified MS, BAP at 2.0 mg/l resulted in more shoots than at 1.0 mg/l (Wakasa, 1989). BAP at 2.0 mg/l for 42 days in agar solidified (Sripaoraya *et al*., 2003) and static liquid MS (Sripaoraya *et al*., 2006), at 2.5 mg/l in phytagel solidified MS (Ko *et al*., 2006; Smith *et al*., 2002) and at 3.0 mg/l in gelrite solidified (Firoozabady and Gutterson, 2003) and in liquid MS medium (Firoozabady and Moy, 2004) was recommended for pineapple multiplication. These different researchers tested the BAP effect on shoot formation under different set of experimental conditions and the different recommendations and different effects of equal concentration of BAP indicated that the optimal BAP concentration depended on the other factors as medium states, speed of agitation, incubation periods, gelling agents and cultivars. However, none of these factors at different levels were tested in combinations with different concentrations of BAP. The optimal BAP for shoot formation of pineapple have not been identified yet and further work is needed to compare the effectiveness of different BAP concentrations on shoot formation in combination with different levels of at least one if not two or three of the other factors involved in multiplication of pineapple.

Combination of BAP and NAA is the most suggested hormone treatment for pineapple multiplication and used more than any other single or combined hormone treatments. In any of the recommended combinations of these two hormones, the concentration of BAP

ranged from 0.2 to 3.2 and that of NAA from 0.001 to 5.0 mg/l. BAP at 0.2 mg/l in combination with NAA at 5.0 mg/l were recommended for leaf culture in gelrite solidified MS (Firoozabady and Moy, 2004) and BAP at 0.25 mg/l was used in combination with NAA at 0.12 mg/l in agar solidified half salt, full organic MS medium (Das and Bhowmick, 1997). BAP at 0.5 mg/l in combination with NAA at 0.001 in agar solidified MS were used for multiplication of pineapple bud culture (Khan *et al*. 2004) and in combination with NAA at 0.5 mg/l in static liquid MS with double glycine content (Mercier *et al*. 2003) for leaf culture of pineapple. BAP at 1.0 mg/l was recommended for combination with NAA at 1.0 mg/l in half strength agar solidified MS for 30 days (Teng, 1997) and with NAA at 0.1 mg/l (Rahman *et al*., 2001) in full strength agar solidified MS medium for 42 days with shoot formation rate of 10 and 25 shoot per explant respectively. Interestingly, although other concentrations of both hormones were tested many of which shared by both researchers, they agreed on that the BAP used at 1.0 mg/l. The different in the NAA concentration of the best combination recommended by them was 10 times and the rate was doubled. Furthermore, contrary to Rahman *et al* (2001) findings, Devi *et al* (1997) reported that in solid MS medium combination of NAA at 0.1 mg/l with BAP at 2.0 mg/l produced better result than in combination with BAP at 1.0 mg/l.

Although, Kofi and Adachi (1993) proved that BAP at 2.25 mg/l in combination with NAA at 0.5 mg/l in liquid MS culture agitated at 70 rpm induced more shoots than when BAP at1.5 mg/l or at other BAP concentrations (0.1, 0.5, 0.6, 0.8, 1.0 and 1.6 mg/l) Firoozabady and Gutterson (2003) on the contrary recommended combination of BAP at 1.5 and NAA at 0.5 mg/l in MS agitated at 80 rpm. The former reported a rate of 35 while the second reported a rate of 12 shoots per explant per 30 days. The three times different in shoot formation rate indicated that a recommendation could not be taken for grant. BAP at 2.0 mg/l in combination with NAA at 2.0 mg/l in liquid static MS (Dewald *et al*., 1988)

with NAA at1.0 mg/l (Mercier *et al.,* 2003) were respectively suggested for shoot and leaf culture and with NAA at 2.0 mg/l (Fitchet, 1990a) and NAA at 0.1 mg/l (Devi *et al*., 1997) in agar solidified MS for shoot culture. Equal concentration of BAP (2.0 mg/l) was recommended by all of them, but the different in NAA concentration was 10 and 20 times and the rate was 13, 4, 9 and 5 shoots per explant in 30 days of incubation. BAP at 2.1 mg/l in combination with NAA at 0.3 mg/l was recommended in liquid (Daquinta *et al*. 1997) and in temporary immersion system (Escalona *et al*. 1999). BAP at 3.2 mg/l in combination with NAA at 0.25 mg/l was recommended for multiplication of pineapple in agar solidified MS supplemented with adenine hemisulfite at 50 mg/l and coconut milk at 10 %, while BAP at lower concentration (0.5 mg/l) and NAA at higher concentration (2.0 mg/l) was recommended for establishment (Singh and Manual, 2000). These differences in the optimal combination and rate indicated that testing of other combinations of hormones, medium strength, and incubation periods could result in even higher multiplication rate. More work still need to be conducted for developing optimal treatment for multiplication of pineapple.

Combination of BAP with other auxins such as IAA, IBA and 2,4-D was also recommended for *in vitro* multiplication of pineapple. IAA at 0.18 (Hirimburegama and Wijesinghe, 1992), 0.5 (Gangopadhyay *et al*. 2005) and 1.0 mg/l (Almeida *et al*. 1997) were recommended in combination with BAP at 2.25, 5.0 and 2.0 mg/l respectively. Gangopadhyay and co researchers suggested that over 4 subcultures the BAP concentration reduced by 50 % at each subculture and Hirimburegama and co researchers noticed that reducing the BAP to 0.23 mg/l blocked the multiplication of shoots. Almeida and co researchers, on the other hand, reported that the optimum concentration of BAP varied among stages and varieties. Keeping IAA constant at 1.0 mg/l, the best establishment and multiplication of Perola pineapple were obtained at 2.0 and 1.0 mg/l of BAP and that of

Primavera obtained at 3.0 and 2.0 mg/l of BAP. All of these recommended combinations of BAP and IAA were tested in agar solidified MS and at fixed IAA level. Testing of different concentrations of IAA and comparison of BAP and IAA in solid, static and agitated liquid cultures and different incubation periods might result in better multiplication rate. Liu *et al.* (1989) recommended 2,4-D at 0.1 mg/l in combination with BA at 0.5 mg/l for multiplication and singly applied 2,4-D at 0.1 mg/l for establishment of bud culture of Red Spanish pineapple. On the other hand, leaf culture of Queen pineapple failed to respond to single application of BAP and 2, 4-D each at 0.5, 1.0, 1.5 and 2.0 mg/l in agarose solidified MS (Sripaoraya *et al*. 2003). However, combination of 2,4-D at 0.5 mg/l with any of the BAP concentrations induced shoot formation with maximum establishment percentage of 85 % of the leaves and formation of 4 shoots per leaf obtained at 2 mg/l of BAP. Combination of 2,4-D and BAP was also used for leaf culture of pineapple but at lower BAP concentration (1.0 mg/l) in agar solidified MS medium (Sripaoraya *et al*., 2001b). Use of IBA in combination with BAP was reported at 1.0 mg/l of each hormone (Boxus *et al*., 1991). Triple hormone combination in which IBA was included was BAP, IAA each at 2.0 mg/l and IBA at1.8 mg/l (Teixeira *et al*., 2006) with rate of 13 shoots per explant in 75 days of incubation. In all cases in which combinations of BAP and NAA were recommended it was neither clearly stated nor supporting data were provided to indicate that other auxins were found inferior. Similar, when auxins other than NAA were recommended in combination with BAP there was no indication that they were better than NAA.

Single application of KN and comparison of KN to BAP is very rare. KN is usually used in combination with one or two auxins (NAA, IBA, IAA) and natural additive as casein hydolysate Using whole small crown, Omokoio *et al.* (2001) tested singly applied KN and BAP at concentrations range of 2 to 10 mg/l in static liquid half strength MS

medium and recommended KN at 6 mg/l over BAP. Hamad and Taha (2003) found that singly applied KN was more effective than Zeatin (ZN) for leaf, bud, and etiolated node culture of Moris pineapple incubated for 60 days. Mathew and Rangan (1979) reported that singly applied KN at 2.1 mg/l resulted in higher shoot formation rate than in combination with either one or both of NAA and IBA. But the percentages of established cultures were less than that of combination of three hormones. Single application of 2iP (6-(γ, γ-dimethylallylamino) purine) for pineapple bud and leaf culture was not reported. However, combination of 2iP at 0.98 mg/l with 2,4-D at 0.9 mg/l was the best treatment for leaf culture (Soneji *et al.*, 2002b) but less effective for bud culture than combination of three hormones KN, NAA and IBA each at 2 mg/l (Soneji *et al*. 2002a). Using of ZN in combination with NAA each at 2 mg/l in agar solidified and liquid MT medium agitated at 100 rpm induced more shoots than using of BA and KN but for cost reason KN was preferred over ZN (Fitchet, 1990a). Kofi and Adachi (1993) reported that the better cytokinine type for combination with fixed concentration of NAA (0.5 mg/l) depended on cytokinin concentrations, pineapple varieties and incubation periods. After 30 days of incubation and at concentration of 0.5, 1.0 and 2.23 mg/l, BAP was better than ZN and 2iP. At concentration of 0.6 and 0.8 mg/l, 2iP was the best and at 0.1 and 1.6 mg/l KN was the best for multiplication of Smooth cayenne. For Sugar loaf, BAP was the best at 0.5 and 1.0 mg/l while at 1.6 and 2.23 mg/l KN was the best and at 0.6 and 0.8 mg/l 2iP was the best cytokinine for combination with NAA at 0.5 mg/l. However, after 60 days of incubation, BAP at any concentrations (0.1, 0.5, 0.6, 0.8, 1.0, 1.6 and 2.23 mg/l) was the best followed by 2iP for multiplication of both Smooth cayenne and sugar loaf. On the other hand, ZN at any concentration and in both incubation periods was the least efficient of all cytokinin types for both varieties.

Since its suggestion three decades ago (Mathew *et al*., 1976), triple combination of KN at 2.1 mg/l, NAA at 1.8 mg/l and IBA at 2.0 mg/l have been repeatedly used in solid MS medium for 42 (Khatun *et al*., 1997), 60 (Mathew and Rangan, 1981; 1979) and 70 days (Soneji *et al*., 2002a) of incubation. The best shoot formation in solid MS enriched with the three hormones ranged from 8 (Mathew and Rangan, 1979) to 25 (Soneji *et al*., 2002a) and 30 shoots (Khatun *et al.*, 1997). The triple combination was also tested in liquid MS agitated at 100 rpm (Soneji *et al*., 2002a; Mhatre and Rao, 2002a, c) for 70 days of incubation. Mhatre and Rao (2002 b) used also agitated culture (100 rpm) but reduced the NAA concentration to 0.1 mg/l and increased incubation to 84 days. The shoot formation was higher than solid and reached 66 shoots per explant. However, testing of this triple hormone combination at different concentrations of either one or two of the hormones reduced the rate to 4 shoots and establishment to 15 % (Khatun *et al*., 1997). Similarly, Liu *et al*. (1989) favored combination of BA at 0.5 mg/l and 2,4-D at 0.1 mg/l over triple combination of KN, NAA and IBA. Rahman *et al.* (2001) reported that in agar solidified MS, double combination of KN and NAA each at 1.0 mg/l for 42 days resulted in higher shoot formation than triple combination of KN, NAA and IBA each at 1.0 mg/l. Mathew and Rangan (1979) kept KN concentration constant at 2.1 mg/l, increased the concentration of NAA to 5.4 mg/l and replaced IBA with IAA at 5.1 mg/l in liquid MS medium agitated at 120 rpm for 50 days and obtained a rate of 63 shoots. Similarly, replacement of NAA with IAA and increasing the concentration of each hormone to 5 mg/l in solid MS medium resulted in formation of 20 shoots per 30 days of incubation (Bordoloi and Sarma, 1993). Instead of KN and NAA, Teixeira *et al*. (2006) used triple combination of BAP, IAA each at 2.0 mg/l plus IBA at 1.8 mg/l in static liquid modified MS and obtained a rate of 13 shoots per explant in 75 days.

With exception of Hirimburegama and Wijesinghe (1992) and Liu *et al.* (1989) who respectively recommended single application of NAA at 0.02 mg/l and 2,4-D at 0.1 mg/l for establishment of primary explants, singly applied auxins were neither recommended for establishment, multiplication nor for callus induction from primary and secondary explants. Dicomba and Picloram were not used singly for shoot formation. However, Dicomba in combination with BAP at higher concentration (Soneji *et al*., 2002b; Daquinta *et al*., 1997) used for induction of callus from leaf base and at lower concentration for regeneration of shoots. Singly applied Picloram at 3.0 mg/l in gelrite solidified MS used mainly for induction and maintaining of embyrogenic callus from leaf explants and in gelrite solidified BAC medium (Firoozabady *et al*., 2006; Firoozabady and Moy, 2004) and agarose solidified MS (Sripaoraya *et al*., 2003) for induction and maintenance of embryos.

Fernando (1986) and Zepeda and Sagawa (1981) suggested respectively MS medium supplemented with coconut milk at 10 % plus NAA at 10 mg/l and coconut milk at 25 % plus BAP at 1.0 mg/l for establishment of lateral buds and coconut milk at 15 % in hormone free MS medium (Akbar *et al*., 2003) used for shoot elongation. Combination of coconut milk at 15 %, Casein hydrolysate at 400 mg/l plus NAA at 10 mg/l in solid MT medium (Fitchet, 1990b) used to induce and maintain callus culture from crown sections and in liquid filter bridge MS medium (Mathew and Rangan, 1981) to induce and maintain callus culture from base of *in vitro* obtained shoots. However, the same combination was also reported for direct shoot formation from crown lateral (Soneji *et al*., 2002a) and from leaf base (Soneji *et al*., 2002b). Liu *et al.* (1989) compared the effect of MS medium enriched with different combinations of coconut milk, Casein hydrolysate and hormones for establishment, multiplication of lateral buds and callus culture and recommended BAP at 0.5 and 2,4-D at 0.1 mg/l for multiplication and BAP at 10 mg/l plus NAA at 4 mg/l for

callus induction. Coconut milk at 10 % plus BAP at 0.5 mg/l and NAA at 2 mg/l (Singh and Manual, 2000) and Casein hydrolysate at 200 mg/l plus KN at 5 mg/l and IBA at 10 mg/l (Bordoloi and Sarma, 1993) were used for multiplication of crown lateral buds. MS medium devoid of hormone, but enriched with combination of Casein hydrolysate at 400 mg/l and coconut milk at 5 % used in phytagel solidified MS medium (Ko *et al*., 2006; Smith *et al*., 2002) and in static liquid filter bridge (Mathew and Rangan, 1981) for regeneration of shoots from callus cultures.

1.2.2. Basal media type and modifications

1.2.2.1. Media type

Full strength MS medium is the most common medium used for establishment and multiplication of pineapple. In spite of this almost unanimous use of MS medium, the MS medium could not be taken for granted as the best medium for multiplication of pineapple without being compared with other MS strength, media types and subject to some medium components modifications. In fact, in just only few cases in which MS was compared with other media types, contradicting results were reported. Bhatia and Ashwath (2002) for instance reported that agar solidified B5 (Gambor medium) enriched with BAP at 1.0 mg/l doubled the shoot formation (7 shoots per explant per month) compared to 4 shoots obtained in MS medium. In the contrary, according to Bordoloi and Sarma (1993) agar solidified MS enriched with NAA, IBA and KN at 5 mg/l each not only was better than B5, but none of shoots in the B5 (Gambor medium) medium established or multiplied. Liu *et al.* (1989), on the other hand, preferred MS medium over B5 and Fitchet (1993, 1990a; b) used MT medium for both stages without providing details. Soneji *et al.* (2002a), Mhatre and Rao (2002a) and Mathew *et al.* (1976) used Nitch medium while Sripaoraya *et al.* (2003) used MT medium and Firoozabady and Gutterson (2003) used mix of MS salt and B5 vitamins for establishment but all recommended MS for multiplication. Mercier *et*

al. (2003) increased glycine to 4 mg/l, kept the same MS microelements but replaced the macro elements by that of Knudson formulation during multiplication while Devi *et al.* (1997) kept the inositol concentration at100 mg/l during establishment but decreased the concentration of inositol to 50 mg/l during multiplication. Modified MS medium made of MS salt and vitamins of White medium (Teixeira *et al*., 2006; Fernando 1986), MS ½ salt and full vitamins (Teng 1997; Das and Bhowmick, 1997), MS half strength (Omokoio *et al.*, 2001; Zepeda and Sagawa, 1981) were used for both stages. No explanations were given for using of modified medium and different media at different stages. Nevertheless, it clearly indicated the importance of medium components for establishment of primary and for shoot formation.

1.2.2.2. Medium states

Inserting the explants in solid medium, placing of the explants over filter paper bridge in liquid medium and directly in thin layer of liquid medium and in liquid medium agitated at various revolution per minute (rpm) were used during multiplication stage. In almost all cases in which solid medium were used, the solidifying agent was agar. However, in few cases phytagel (Ko *et al*., 2006), gelrite (Firoozabady and Gutterson, 2003) and agarose (Sripaoraya *et al*., 2003) were also suggested. Contradicting findings were reported indicating that the medium state may not a decisive factor of *in vitro* shoot formation of pineapple. Equal rate of shoot formation (4 shoots per 42 days) obtained in solid medium (Khan *et al*., 2004), solid and filter paper bridge cultures (Escalona *et al*., 1999) and in cultures agitated at 120 rpm for 30 days (Zepeda and Sagawa, 1981). Equal rate of 7 shoots per 42 days obtained in solid and filter paper bridge (Fernando, 1986) and in solid and cultures agitated at 120 rpm (Aydieh *et al*., 2000) and equal rate of 9 shoots per 30 days obtained in solid and shaking at 120 rpm for 30 days (Fitchet, 1990a) and from shoots that were directly placed in static liquid culture for 60 days (Be and Debergh, 2006). A shoot

formation rate of about 10 shoots per explant obtained in medium solidified with gelrite (Firoozabady and Gutterson, 2003), phytagel (Ko *et al*., 2006) and agarose (Sripaoraya *et al*., 2003). Kofi and Adachi (1993) reported 35 shoot per explant on cultures agitated at 50 rpm for 30 days and equal rate of shoot formation (35 shoots per explant) obtained also in agar solidified MS but after 70 days of incubation (Soneji *et al*., 2002b).

On the contrary, Mathew and Rangan (1981, 1979) reported that filter bridge culture triple the shoot formation and agitation at 120 rpm increased shoot formation 10 times compared to 6 shoots per 50 days of incubation obtained in agar solidified MS medium while Fernando (1986) obtained equal rate in solid and filter paper bridge but agitation the cultures at70 rpm for 42 days increased the shoot formation rate 10 times. Soneji *et al*. (2002a) reported that after 70 days of incubation, using of filter bridge and shaking at 100 rpm resulted in 6 time more shoot than solid culture. Firoozabady and Gutterson (2003) reported that the total shoots obtained after 7 cycles of multiplication of intact shoots in liquid cultures was two times as that of gelrite solidified medium while Almeida *et al.* (2002) reported that the total shoots obtained after 5 cycles of multiplication of intact shoots in static liquid were 18 times higher and that of halved shoots was only 4 times higher than total shoots obtained in agar solidified MS medium. Liquid was super than solid medium, However, it is interesting to note that the data reported by Almeida *et al.* (2002) showed that the magnitude of difference between the two medium states depended on explants intactness and BAP concentration. At different concentrations, total from intact shoots in liquid medium containing BAP at 1.5 and 3.0 mg/l was 18 and 37 times more than solid while that obtained from halved shoot was 4 times more than solid medium at both BAP concentrations. The effectiveness of the medium states, BAP concentrations and explants manipulation on the shoot formation capacity of the explants were interdependent on each other. Halving the explants either reduced the effect of liquid or improved the

effect of solid medium. Hence, keeping the medium state constant during testing the effect of hormone on the shoot formation and the hormone constant during testing the effect of medium states, as it is usually done, would not reflect the potential of the medium states and the different concentrations of the hormone. Investigation of other factors as medium strength, volume per culture, pH and sucrose might not only change the magnitude of the medium states and hormone effect but may even reverse it. Unfortunately, in all of the reported pineapple tissue cultures all of these factors were always kept constant. Optimization of the shoot formation using static liquid culture by investigation the effect of medium strength, volume per culture, pH, sucrose and testing of explants density and size individually and in several combinations with each others should be given the first priority. Static liquid cultures if not better than solid and agitated cultures in term of shoot formation is definitely the cheapest.

1.2.2.3. Sucrose content and pH adjustments

In both solid and liquid cultures the medium is most commonly supplemented with sucrose at 30 g/l and adjusted to pH 5.7 before being autoclaved. In few cases, sucrose at 20 g/l were used in phytagel solidified MS (Ko *et al*., 2006; Smith *et al*., 2002), static liquid (Hamad and Taha, 2003) and liquid MS medium agitated at 100 rpm (Soneji *et al.*, 2002a; Mhatre and Rao, 2002a; b). Sucrose at 35 g/l used in liquid MS agitated at 70 rpm (Kofi and Adachi, 1993). Almeida *et al.* (1997) used agar solidified MS medium enriched with sucrose at 40 g/l for both stages while Sripaoraya *et al.* (2003) suggested MT enriched with sucrose at 50 g/l during establishment and MS medium and sucrose at 30 g/l during multiplication. Adjustment of the medium pH to 6.0 during establishment (Broomes and McEvan, 1994) and pH 5.0 (Fitchet, 1990a) and pH 6.0 (Teixeira *et al*., 2006) during multiplication were also reported. Escalona *et al.* (1999) reported that the pH of the pineapple multiplication medium decreased over incubation to reach equilibrium of 3.5 by

the seventh week. Attaining of equilibrium matched with the highest shoot formation rate per week. Both occurred at the seventh week. These different recommendations of sucrose and pH adjustments indicated that optimization of establishment and multiplication could not be obtained unless the effect of these two factors on establishment and multiplication were tested individually and in combinations of both. Different concentrations of sucrose and pH values were used in separated experiments but, neither the effect of different sucrose concentrations nor medium pH values on the explants establishment and *in vitro* shoot formation were compared. Sucrose is the most heavily used component of the medium and very important item of cost. On large scale propagation using of the lowest possible sucrose enrichment would result in substantial saving in the cost of sucrose.

1.2.2.4. Medium volume and strength

A volume of 10 (Hamad and Taha, 2003; Fitchet, 1990b), 12.5 (Akbar *et al*., 2003), 20 (Ko *et al*., 2006; Teixeira *et al*., 2006; Firoozabady and Moy, 2004; Soneji *et al*., 2002a; Fitchet, 1990a), 25 (Perez *et al*., 2009; Sripaoraya *et al*., 2003; Soneji *et al*., 2002b; Aydieh *et al*., 2000; Perez *et al*., 2003; 2004), 30 (Be and Debergh, 2006; Firoozabady and Gutterson, 2003, Mercier *et al*., 2003; Sripaoraya *et al*., 2001a), 40 (Akbar *et al*., 2003; Sripaoraya *et al*., 2001b) and 50 ml (Sripaoraya *et al*., 2006; 2003 Soneji *et al.*, 2002a; Mhatre and Rao, 2002b,c) per culture were reported. The minimum volume of medium per culture was 7 ml of solid MS during establishment (Fitchet 1993, 1990a) and the maximum volume was 250 ml (Escalona *et al*., 1999). Most of the time, 15 to 20 ml per culture were used during both of establishment and multiplication stages. However, in all cases, the effect of different medium volumes per culture on the shoot formation was not investigated. In temporary immersion system and density of 5 clusters of 3 shoots, Escalona *et al*. (1999) reported that using of 200 ml of medium per explant resulted in the highest shoot formation. It is surprising that in conventional pineapple system, medium

volume was not only ignored as a factor but also was rarely reported. Beside it reflects the amount of nutrient and hormones provided for single explant, the medium volume dispensed per culture is very important for cost estimation and need to be optimized particularly in relation with medium state, pH and explants density. Dispensing the medium at smaller volume per culture would increase the number of vessels and consequently the operation time of laminar, shelving space, the electricity cost of incubation period. Cost estimation could not be made if the medium volume per culture is not known and would be different at different media strength and different content of sucrose. So far the assessment of different shoots multiplication treatments is based in shoot formation rate and total of shoots over consecutive subcultures. Using of shoot formation rate per shoot per unit volume of medium would make the assessment serve both physiological investigation and cost analysis goals.

Several modification of MS medium was proposed for both of establishment and multiplication stages. In some of these modifications the MS diluted to half strength and on other one of the MS medium component completely replaced by component of other type of media. Khan *et al.* (2004) compared full and half strength MS during pineapple multiplication and Perez *et al.* (2004) during elongation stage. Both recommended MS at full strength. Half strength of liquid MS (Omokoio *et al.*, 2001; Zepeda and Sagawa, 1981) and half strength salt but full organic of solid MS (Das and Bhowmick, 1997; Teng, 1997) were suggested for both stages. Combination of full salt of MS and vitamins of White medium in agar solidified medium (Fernando, 1986) and MS and vitamins of B5 medium in gelrite solidified medium (Firoozabady and Gutterson, 2003), Nitch medium (Soneji *et al*., 2002a; Mhatre and Rao, 2002a; Mathew *et al*., 1976) and MT medium (Sripaoraya *et al.* (2003) were used for establishment but all recommended MS for multiplication. On contrary, Teixeira *et al*. (2006) used combination of full salt of MS and vitamins of White

medium in liquid state for multiplication stage. Doubling of glycine of liquid MS medium were used for shoot bud culture (Mercier *et al*., 2003) and etiolated node culture (Souza *et al*., 2003) and combination of macro elements of Knudson and microelement of MS medium (Mercier *et al*., 2003) for leaf culture. Devi *et al.* (1997) kept the inositol concentration at100 mg/l during establishment but decreased the concentration of inositol to 50 mg/l during multiplication.

No explanations were given for using of modified medium or different media at different stages. Nevertheless, it clearly indicated the importance of medium components for establishment of primary and for shoot formation. These modifications of MS indicated that different levels of the same component and same level of the different components of MS medium had different effects on the shoot formation rate. Medium strength had been reported to effect in rooting response, however, little attention was paid to the effect of strength in establishment and multiplication. With exception of Khan *et al.* (2004) who reported that dilution of MS to half strength reduced the rate and length of shoots compared to full strength MS medium, medium strength was never compared. For highest multiplication not only the strength of medium as whole but also the effect of different strength of the various medium components as groups and at individual compound need to be investigated and optimized. Since experiment of this kind is too big to be handled, optimization of medium strength as whole, sucrose at different pH, volume per culture and medium state is key step from which further investigation of the different component could be followed.

1.2.3. Explants size and density

At each multiplication cycle a multiple-shoots complex developed from each single explant. Depending on the state and strength of the multiplication medium, hormone treatments and incubation periods the number of shoots per multiple-shoots complex

ranged from 4 (Khan *et al*., 2004) to 76 shoots (Fernando, 1986). An average, shoot length of 10 (Hamad and Taha, 2003), 30 (Aydieh *et al*., 2000) and 60 (Soneji *et al*., 2002b), 95 mm (Khan *et al*., 2004) and different shoot length ranged from 5 up to 100 mm (Escalona *et al*., 1999) were reported during multiplication. Graded into small, intermediate and long shoots, the percentage of each grade varied among different medium strength (Khan *et al*., 2004; Vesco *et al*., 2001) and different hormone treatments and medium states (Fitchet, 1990 a). Dewald *et al.* (1988) reported that few long and many small shoots were formed at each cycle of multiplication and the frequency of longer shoots increased over subcultures. Generally at each and over consecutive multiplication cycles over 50 % of the shoots were shorter than 15 mm in length. However, in almost all of the reported *in vitro* multiplication of pineapple 20 mm long shoots were selectively chosen and used at density of one intact shoot per culture. In all studies of multiplication, the effect of shoot size in shoot formation was not investigated and in almost all the frequency of shoot of different length was not reported. Unless it proven that the shoot size have no significant effect on shoot formation capacity, shoot formation rate of selective shoot size did not reflect valid comparison of different multiplication treatments. Different treatments could result in equal shoot formation rate and equal average of shoot length but different frequency of the shoot of equal length. As the shoot length may affect its shoot formation capacity, the total shoots produced in the next cycle from treatments with equal shoot formation rate and different frequency of shoot length would be different. In fact, Firoozabady and Gutterson (2003) mentioned that when explants longer than 20 mm were used the multiplication was 45 fold while when shorter shoots used the multiplication was only 7 fold. Beside their differences on the shoot formation capacity, shoots of different size may behave differently at same explants density, medium volume per culture and at different combination of explants density and medium volume per culture.

During multiplication in conventional system, at each subculture the multiple-shoots complex either transferred as whole (Almeida *et al*., 2002), cut into quarters (Boxus *et al*., 1991), clusters of 2-3 shoots (Firoozabady and Gutterson, 2003; Escalona *et al*., 1999), separated into individual shoots and halved (Almeida *et al*., 2002; Bhatia and Ashwath, 2002; Teng, 1997; Mathew and Rangan, 1979) or kept intact and subculture at density of 1 explant (Teixeira *et al*., 2006; Sripaoraya *et al*., 2003; Bhatia and Ashwath, 2002), 2 (Be and Debergh, 2006; Soneji *et al*., 2002a) 3 (Hamad and Taha, 2003), 4 (Khan *et al*., 2004) and 5 (Perez *et al*., 2009) shoots per culture into fresh medium for the next cycle of multiplication. In bioreactor system density of 5 clusters each consisted of 2-3 shoots (Firoozabady and Gutterson, 2003; Perez *et al*., 2004, 2003; Escalona *et al*., 1999) and 50 clusters (Firoozabady and Gutterson, 2003; Escalona *et al*., 1999) were used per bioreactor vessel. Halving of individual shoot resulted in higher rate and total shoots than intact shoot particularly on liquid medium (Almeida *et al*., 2002). However, the effect of explants density, size and medium volume per culture on shoot formation in conventional system was not investigated. All of these factors not only could effect the shoot formation per explant but also using of higher density and larger medium volume per culture would reduce the vessels and shelving space and consequently the operation time of laminar and electricity cost of incubation period.

The best explants density is that which resulted in the highest rate per explant at lowest medium volume per culture. However, using of lower medium volume per culture would increase the the number of required vessels and shelving space and consequently the cost. Increasing the explants density and decreasing medium volume per culture is expected to decrease the shoot formation per explant. However, it would increase the total shoots per unit of medium volume. An explants density which might be rejected because of lower rate of shoot formation could be the best density for higher total of shoot production per unit of

medium volume and vice versa. A compromise between higher shoot formation rates per explant and medium volume with lowest number of vessels (highest medium volume per vessel) should be made. For better judgment, the explants density and medium volume per culture should be tested in combination of both factors. It could not be rolled out that if the two factors explants density and medium volume tested at different levels, different explants density may have different optimal of medium volume. In addition, the explants density at which the shoot formation decline may vary according to explant size. To maintain optimal rate, it may necessary to use different explants density per culture for shoots of different size. For proper assessment of different multiplication treatments the rate per explant as well as per unit of medium volume should be both considered during treatments assessment. In bioreactor system, at fixed density of 5 clusters the higher rate of shoot formation after 42 days of incubation (23 shoots per explant) was obtained using 1000 ml of medium (Escalona *et al*., 1999). That is 200 ml of medium per one explant. In other word production of one shoot required using of 9 ml of medium (200 ml/ 23 shoots). Including only the cost of medium, the cost of one shoot exceeded 7 cents. If all other items of cost were included the cost of one shoot would be extremely high and commercial application of this system is out of question. Comparing density of 50 and 25 clusters per 10-liters bioreactor (fixed volume of medium) after 4 and 7 weeks of incubation showed that high density and short incubation resulted in higher total shoots but shorter shoots than low density and long incubation.

In all of the reported studies of pineapple multiplication the assessment of different treatments was based in shoot formation rate and total shoot production without considering the frequency of shoots of different length. Since shoots of different size would probably result in different rate of shoot formation, correct expectation of rate and total of shoots per cycle and over several cycles could not be made unless the frequency of

shoot size and its shoot formation is known. Furthermore, shoot formation per unit of medium volume as well as cost assessment was completely ignored. A compromise between low total cost and low cost per unit shoots and higher rate per explant and total shoots might favor one procedure over the other but such possibility was not investigated. Hence the applicability of all of these recommended treatments and protocols particularly for mass production of propagules need to be rechecked.

1.2.4. Incubation periods and subcultures

The proper length of incubation period should be determined based on objectives and management reasons. For propagators, the incubation period should be extended till the maximum shoots formation per explant is obtained and subculturing continuously repeated till the shoot formation per explant per subculture sharply declined or the target total of shoot production obtained. For physiologist, incubation period may terminate when the rate of shoot formation, shoot length and weight per explant per unit of time start to decline and the medium is no longer supportive of highest growth rate. However, in all previous studies the length of incubation period were arbitrarily picked and kept fixed. Incubations for different periods were not compared and the rate of shoot formation was reported as average of several subcultures rather than per each subculture. Firoozabady and Gutterson (2003) reported a repeated cyclic procedure of 15 days long cycle on liquid static and solid medium while Fernando (1986) replaced the medium of liquid culture agitated at 70 rpm every 21 days for two times before counting the shoots after 42 days of incubation. Incubation length of 30 days and repeated subculturing in liquid medium for 3 (Fitchet, 1990a), 4 (Zepeda and Sagawa, 1981), 5 (Almeida *et al*., 2002), 7 (Firoozabady and Gutterson, 2003), 11 (Kofi and Adachi, 1993) and 13 multiplication cycles (Dewald *et al*., 1988) and in solid medium for 4 (Gangopadhyay *et al*., 2005), 5 (Almeida *et al*., 2002; Devi *et al*., 1997; Bordoloi and Sarma, 1993), 6 (Singh and Manual, 2000) and 7

multiplication cycles (Firoozabady and Gutterson, 2003) were reported. Incubation for 42 days and multiplication for 4 cycles (Perez *et al*., 2004; 2003; Aydieh *et al*., 2000) in liquid medium and for 4 (Perez *et al*., 2009**),** 6 (Bhatia and Ashwath, 2002), 8 (Rahman *et al*., 2001) and10 cycles (Teng, 1997) in solid medium were reported, but the rate was also presented as average per explant over several subcultures. Of all only Fitchet, (1990a) and Kofi and Adachi (1993) reported the rate while Devi *et al.* (1997) reported the total at each subculture. The shoot formation after incubation for 35 (Khatun *et al*., 1997), 42 (Khan *et al*., 2004; Sripaoraya *et al.,* 2003; Escalona *et al.,* 1999), 50 (Mathew and Rangan, 1981, 1979), 60 (Be and Debergh, 2006; Mathew *et al*., 1976), 65 (Omokoio *et al*., 2001), 70 (Soneji *et al*., 2002a; Mhatre and Rao, 2002a and c; Hirimburegama and Wijesinghe, 1992), 75 (Teixeira *et al*., 2006) and 84 days (Mhatre and Rao, 2002b) were reported without elaboration of how many times the shoots were subcultured.

Testing the effect of different incubation periods on the shoot formation rate per explant is very important for selection of optimal hormone type and concentration, cost and management of multiplication stage and for understanding the physiology of *in vitro* shoot formation. For instance, Kofi and Adachi (1993) reported that either BAP or KN induced highest while 2iP induced the lowest shoot formation of sugar loaf pineapple incubated for 30 days. However, if incubated for 60 days, BAP resulted in highest shoot formation followed by 2iP while KN dropped to become the least effective hormone. Irrespective of hormone types, incubation for 60 days resulted in formation of more shoots than incubation for 30 days. Over 11 subcultures on liquid MS enriched with BAP at 2.23 and NAA at 0.5 mg/l and agitated at 50 rpm, the rate was increased from 15 shoots at the first to 55 at the seventh and decline afterward to 38 shoots by the eleventh subculture. Singh and Manual (2000) reported that over 6 subcultures, each was 30 days long in agar solidified MS enriched with combination of BAP at 3.2 mg/l and NAA at 0.25 mg/l, the

shoot formation increased over the first four subcultures from 5 shoots at the first to 7 shoots at the fourth and declined by the sixth subculture to 4 shoots per explant.

Many articles have been published and shoot formation rate ranged from 4 shoots per 30 days to 76 shoots per 42 days of incubation and several estimations of total shoots per year ranged from 280 (Devi *et al*., 1997) to 100 000 shoots (Sripaoraya *et al*., 2003) were reported, yet *in vitro* culture of pineapple could not be considered as a well established technique. More investigation of establishment and multiplication stages and refinement of findings still need to be done before an optimal universal treatment could be claimed. There are many factors that could effect multiplication. All except hormones and medium states which were only partially investigated were ignored or received very little attention. The effect of factors as medium strength, sucrose concentration, pH, medium volume dispensed per culture, explants density per culture, explants size and age (number of subcultures) and incubation periods was neither investigated individually nor in combination with other set of factors. In each single experiment these factors were arbitrarily chosen and kept constant. Beside their expected effect on shoot formation, these factors are very important for cost estimation and management of micropropagation system. Furthermore, estimation of yearly production of shoots was just an extrapolation that extended to cover period not supported by actually conducted cycles of multiplication. Yet surprisingly, some claimed commercial production while on the same time none provide an estimate of cost per shoots, per subculture and even a roughly estimated total of cost. In summary, induction of *in vitro* multiplication of pineapple and potential of mass production of propagules is well documented but optimization of establishment of primary explants and multiplication is very far from being reached and commercial production using any of the reported protocols is questionable.

1.3. *In vitro* rooting of shoots

Shoots produced during multiplication stage either reused for the next cycle of multiplication, transferred to *in vitro* rooting or to an elongation medium. *In vitro* rooting of pineapple could be obtained in hormone free and hormone contained full strength agar solidified and half strength liquid MS medium. Other media type as White and MT was also in few cases used for rooting of pineapple. The main hormones used for rooting were IBA and NAA. However, not only the researchers disagree in which of these two hormones is the best for rooting and whether single or combination of the two hormones resulted in best rooting but also even those who recommended single application of one of these hormones and those recommended combined application disagree in the optimal concentration. Furthermore, comparing the published results indicated contradicting effectiveness of the same concentration of the hormone shared by different researchers.

1.3.1. Hormones and Media

1.3.1.1. Hormone free medium

Over 28 % of the reported *in vitro* rooting of pineapple was done in hormone free agar solidified full strength MS medium. However, almost at all time in which the rooting was done in hormone free medium, the results were presented as general statement without providing rooting data or comparison with hormone contained medium. Solid half strength and liquid full strength MS were less frequently used for rooting while half strength hormone free liquid medium, on the other hand, was exceptionally left without being tried. In few cases, hormone free medium was proven to be as good as hormone contained. Be and Debergh (2003), for instance, obtained equal rooting response in full strength liquid MS hormone free and enriched with NAA at 1.0 mg/l and suggested MS hormone free for rooting. Aydieh *et al.* (2000) reported that all shoots rooted in solid MS hormone free and

enriched with singly applied IBA and NAA at 0.5, 1.0 and 2.0 mg/l and Soneji *et al.* (2002 a) reported that all shoots rooted in solid and liquid MS and White medium hormone free and enriched with combination of IBA at 0.4 mg/l and NAA at 0.2 mg/l. However, addition of hormone induced more and longer roots than that on hormone free medium. On contrary, Bhatia and Ashwath (2002) reported that only 30 % of the shoots rooted in hormone free full strength agar solidified MS medium, 50 % rooting in medium enriched with IBA at 0.5, 1.0 and 2.0 mg/l while 65 % rooting obtained using combination of IBA and IAA each at 2.0 mg/l.

1.3.1.2. Hormone contained medium

Generally, full and half strength agar solidified MS medium enriched with auxins particularly IBA and NAA singly and in combination were the most commonly used root inducing treatments. However, the researchers who used the same hormone type recommended different concentrations. Singly applied IBA was recommended for rooting of pineapple at 0.5 (Dolgov *et al*., 1997; Fernando, 1986), 1.0 (Khan *et al*., 2004) and 2.0 mg/l (Devi *et al*., 1997; Bordoloi and Sarma, 1993; Akbar *et al*., 2001) and singly applied NAA was recommended at 1.0 (Fitchet, 1993, 1990 a) and 2 mg/l (Aydieh *et al*., 2000). Similar, combination of IBA and NAA at equal concentration of 0.1 mg/l (Teng, 1997), 0.2 mg/l (Rahman *et al*., 2001), 0.5 mg/l (Firoozabady and Gutterson, 2003) and 1.0 mg/l (Singh and Manual, 2000) and at different concentrations, IBA at 0.4 mg/l and NAA at 0.18 mg/l (Soneji *et al*., 2002 b; Khatun *et al*., 1997; Mathew and Rangan, 1979) and IBA at 0.4 and NAA at 0.05 mg/l (Mathew and Rangan, 1981) were also recommended for rooting of pineapple. Others kept the IBA at 0.4 mg/l but replaced NAA with IAA at 0.13 mg/l (Kofi and Adachi, 1993) while other increased the IBA to 2.0 mg/l and replaced NAA with KN at 0.4 mg/l (Gangopadhyay *et al*., 2005). Bhatia and Ashwath (2002) and Teng (1997) reported respectively that after the fifth subculture, 50 % of the shoots rooted in

multiplication medium consisted of agar solidified full strength B5 medium enriched with BAP at 2 mg/l and half strength MS medium enriched with combination of BA and NAA each at 0.1 mg/l.

Comparing the results reported by different researchers indicated that the concentration recommended by one as the best was found by the other as the least effective and the treatment which was the best for root induction (rooting percentage) was less effective for root formation (root number per explant) and elongation (root length). Using local cultivar, Khan *et al*. (2004) obtained 100 % rooting in medium enriched with IBA at 0.5 and at 1.0 mg/l but IBA at 1.0 mg/l resulted in more and longer roots. Aydieh *et al*. (2000) obtained 100 % rooting of Queen pineapple in medium enriched with IBA and NAA singly applied at 0.5, 1.0 and 2.0 but NAA at 2.0 mg/l produced more and longer roots than the other concentrations. On the contrary, Devi *et al*. (1997) reported that IBA at 2.0 mg/l resulted in better rooting of Queen pineapple than IBA at 0.5, and 1.0 mg/l. Akbar *et al.* (2001) obtained 100 % rooting of Madhupur cultivar in IBA at 2.0 mg/l while the cultivar failed to root in medium enriched with IBA at 1.0 mg/l and in medium enriched with NAA at 1.0 and 2.0 mg/l and enrichment with combination of NAA and IBA each at 2.0 mg/l reduced the rooting to 50 %. Bhatia and Ashwath (2002), on the other hand, reported that IBA at 0.5, 1.0 and 2.0 mg/l resulted in very low rooting percentage (less than 50 %) of Smooth cayenne. In response to IBA at 1.0 mg/l, Devi *et al.* (1997) and Aydieh *et al.* (2000) both reported 100 % rooting and 6 root per explant, however the different in root length was as much as 6 times (12 and 75 mm respectively). IBA at1.0 (Khan *et al*., 2004) and at 0.2 mg/l (Rahman *et al*., 2001) resulted in equal root number (5 roots per shoot) and equal root length (35 mm). In response to NAA at 1.0 mg/l, Khan *et al.* (2004) and Aydieh *et al.* (2000) both reported 100 % rooting but the different in number of roots per shoot was two

times and root length three times while Be and Debergh (2006) obtained equal rooting response in hormone free and NAA at 1.0 mg/l.

Combination of IBA and NAA at 1.0 mg/l each in half strength agar solidified MS medium resulted in better rooting than single and combined application of IBA and NAA at 0.5 and 1.5 mg/l (Singh and Manual, 2000). The same hormones, but at ten times lower concentration (IBA and NAA each at 0.1 mg/l) was used in full strength solid MS medium for rooting (Teng, 1997). Rahman *et al.* (2001) found that in agar solidified full strength MS, NAA singly applied at 0.2 mg/l induced higher rooting percentage and more roots than singly applied IBA at 0.4 mg/l, but combination of the two hormones (NAA at 0.2 and IBA at 0.4 mg/l) resulted in better rooting (100 % rooting, 8 roots and 35 long roots). On the other hand, lowering the concentration of either one or both hormones to 0.1 mg/l or increasing to 0.5 mg/l reduced the rooting percentage to 50 %. However, the root length remained the same at all concentrations. Using the same combination (IBA, 0.4 and NAA at 0.2 mg/l) in liquid full strength White medium, Soneji *et al.* (2002a) obtained same result (100 % rooting, with 8 roots) but longer roots (42 mm) while in solid half strength MS and same hormone treatment Khatun *et al.* (1997) obtained more (12) and longer (63 mm) roots. Khatun *et al.* (1997) found that decreasing the concentration of both hormones by 50 % (NAA at 0.1 and IBA at 0.2 mg/l) resulted in equal root number per shoot but increased the root length while increasing the concentration by 50 % (NAA 0.4 and IBA 0.6 mg/l) reduced the root number and length. But all of the concentrations resulted in 100 % rooting. On all of these *in vitro* rooting studies of pineapple either different cultivars, medium states, strength or incubation period were used and the different results indicated that factors other than the hormones was very important for rooting and control the effectiveness of the hormones as rooting factor. At different set of factors there would be different optimal hormone types and concentrations.

1.3.1.3. Media type, strength, state and sucrose

In vitro rooting of pineapple is most commonly done in agar solidified MS medium most often in full strength. However agar solidified half strength and liquid MS at full and half strength were used in some cases. Rooting of pineapple obtained also in White medium full strength, agar solidified (Mathew and Rangan, 1981) and liquid (Soneji *et al*., 2002a) and in MT medium full strength agar solidified (Fitchet, 1990a). On the contrary, Bordoloi and Sarma (1993) reported that rooting could not be obtained in SH and White medium enriched with IBA at 2.0 mg/l while good rooting occurred in MS and B5. Comparing of MS and White media each enriched with sucrose at 10 g/l showed that liquid White was superior, inducing more, longer and higher rooting percentage than MS. However, at 20 g/l of sucrose, White medium induced also more roots and higher rooting percentage but the root length in both media were not different (Soneji *et al*., 2002a). On both sucrose enrichment, the strength of liquid MS did not affect the root number per shoot.However, the medium strength effect on the rooting percentage and root length depended on the sucrose content of the medium. At 10 g/l of sucrose, equal rooting obtained in full and half strength MS, while at 20 g/l of sucrose half strength MS induced higher rooting percentage but shorter roots than full strength MS.

Although sucrose is always added to any salt formulation during multiplication and rooting, little attention was paid to sucrose effect on both stages. In most cases, sucrose was added at 30 g/l. However, 10 (Soneji *et al.*, 2002a,b), 20 (Devi *et al*., 1997), 35 (Kofi and Adachi, 1993) and 40 g/l (Almeida *et al.*, 1997) were also used during *in vitro* rooting. Be and Debergh (2003) obtained equal rooting responses, plantlet height and weight at 30 and 40 g/l of sucrose. Soneji *et al.* (2002a) noticed the rooting response was result of interaction between sucrose content, medium strength and hormones. In full strength hormone free and hormone contained medium, 10 g/l of sucrose induced higher rooting

percentage and more roots than 20 gm/l but of equal root length. On the contrary, in half strength hormone free enriched with sucrose at 10 g/l induced higher rooting percentage than that enriched with sucrose at 20 g/l but of equal number and length while in hormone contained equal rooting percentage obtained in both of the sucrose enrichments but sucrose at 10 g/l resulted in more and longer roots than sucrose at 20 g/l. As the different in the reported amount of sucrose used during rooting is too wide ranging from 10 up to 40 gm/l and the medium strength effect depended on sucrose content, hormone and medium state, not only more work is needed to optimize sucrose content and medium strength for *in vitro* rooting but also chance for reducing the cost of medium and sucrose by one fourth does exist and worth pursuing.

1.3.2. Explants size and density

At each multiplication cycle, shoots of various sizes ranging from 10 to 100 mm in length were reported. An average shoot length of 10 **(**Hamad and Taha, 2003), 30 (Aydieh *et al*., 2000), 60 (Soneji *et al*., 2002b), 90 (Be and Debergh, 2006) and 95 mm (Khan *et al*., 2004) were obtained during multiplication. Even if the shoots irrespective of its size are capable of rooting when transferred to hormone free or medium enriched with rooting hormone, the rooting responses of shoots of different size is expected to be different and each may have different optimal rooting treatment for one or all of the rooting parameters. Also some of the shoots particularly longer shoots may not need to be *in vitro* rooted and could easily *ex vitro* rooted. Yet in all rooting studies shoots longer than 25 mm were selectively used for rooting experiments at density of one explant per culture. Surprisingly no comparison of the rooting capacity of shoots of different size under constant or different combinations of factors was reported and the volume of medium per culture was not even mentioned.

The *in vitro* rooting is costly stage and its conduction is justified based on the assumption that it will increase the plantlets survival of acclimatization stage. However, Escalona *et al*. (1999) demonstrated that survival of shoots transferred from multiplication directly to *ex vitro* rooting and acclimatization conditions depended on the shoot length. None of the 20 mm long shoots survived while 40, 60, 70, 80 and 100 % of the 30, 50, 70, 90 and 110 mm long shoots survived *ex vitro* acclimatization respectively. Fitchet (1990a) and Dewald *et al*. (1988) respectively mentioned that rootless shoots longer than 25, 30 and mm could survive hardening and *ex vitro* acclimatization. Fernando (1986) mentioned that shoots longer than 50 mm could be *ex vitro* rooted and acclimatized particularly if the shoots were multiplied in agar solidified medium. Since in all reported rooting studies shoots longer than 25 mm were used for rooting, it is clear that the shoots which have to be *in vitro* rooted to survive acclimatization were left out of testing and the rooting of those in which the *in vitro* rooting could possibly bypassed were tested. The effect of explants density, on the other hand, on the *in vitro* rooting and plantlets survival was not investigated. Be and Debergh (2003) said that 5 shoots per culture were used during *in vitro* rooting on full strength liquid MS and Firoozabady and Gutterson (2003) mentioned that tray with half strength MS medium were used for rooting of shoots of different size. On all other reports one shoot per culture was used and the medium volume per culture was not reported. As long as the effect of explants density, medium volume per culture and shoot length on rooting is not investigated, the rooting treatment could not be optimized and the cost of rooting could neither be estimated nor minimized.

1.3.3. Shoots age and incubation periods

Shoots used for *in vitro* rooting could be taken from any cycle of multiplications (subculture), first, second and the last subculture and after being incubated in the multiplication medium for 30 to 90 days. Shoots of different age (different subcultures and

incubations period) may have different internal hormone balance, rooting ability and sensitivity to externally applied rooting hormones type and concentration and consequently different optimal rooting treatment. In fact, Teng (1997) reported that the range of effective concentration of IBA and NAA combination as root inducing hormone become wider over subcultures and Bhatia and Ashwath (2002) reported that after 5 subcultures 50 % of shoots developed roots in multiplication medium enriched with BAP at 2.0 mg/l. Except in these two reports, the number of subculture (age of shoots) from which the shoots used for rooting was not mentioned.

Emerging of *in vitro* roots was observed after 7 to 10 days of incubation in the rooting medium (Soneji *et al*., 2002 a; Khan *et al*., 2004; Rahman *et al*., 2001; Devi *et al*., 1997) and 100 % rooting could be obtained in 21 days (Firoozabady and Gutterson, 2003). Different incubation periods for 30 (Be and Debergh, 2006; Firoozabady and Gutterson, 2003; Bhatia and Ashwath, 2002; Almeida *et al*., 2002; Soneji *et al*., 2002b; Singh and Manual, 2000; Fitchet, 1990), 42 (Perez *et al*., 2009; Khan *et al*., 2004; Akbar *et al*., 2003), 60 (Gangopadhyay *et al*., 2005; Almeida *et al*., 1997; Mathew and Rangan, 1979) and 70 days (Soneji *et al*., 2002a; Mhatre and Rao, 2002a) was reported. However, in all of these reports the length of incubation period was arbitrarily chosen, kept constant and the effect of different incubation periods was not compared. Even if the rooting percentage and root number may not change significantly after the first 30 days of incubation, longer incubation would result in longer root, taller and heavier plantlets and would certainly increase the electricity bill. In most of these reports, over 80 % of the plantlets survived acclimatization under different acclimatization treatments and conditions. Unfortunately, the relation between different incubation periods, plantlets quality during rooting and survival and growth during acclimatization were not investigated. For highest survival and lowest cost it is very essential that the minimum level of the most crucial plantlet quality

for 100 % plantlets survival be determined and the incubation should not be terminated unless the essential rooting parameter and plantlet quality for survival of acclimatization obtained.

1.3.4. Rooting quality and acclimatization

Assessment of rooting treatment is usually based on rooting percentage, root number per explant, and root length. If the goal is studying the physiology of rooting, monitoring of the changes of these rooting parameters at different treatments in connection with chemical and histological analysis could explain some basis of the physiological process of induction, development and growth of rooting. However, since the propagator goal is plantlets survival during acclimatization the necessity of rooting for survival have to be proven and the relationship between the different rooting parameters and survival need to be determined. *In vitro* rooting is a costly stage. If it does not serve the goal it should not be conducted and if needed it should be assessed using the parameter that has the highest correlation with plantlets survival. Surprisingly, such relationships were not investigated. Escalona *et al*. (1999) demonstrated that shoots could be directly transferred from multiplication medium to *ex vitro* acclimatization provided that the shoots were of proper shoot length. In other word the rooting percentage, root number and length are not essential for survival of acclimatization and instead of *in vitro* rooting the shoot could be transferred to an elongation stage. In fact, elongation stage was suggested by Firoozabady and Gutterson (2003) and Escalona *et al.* (1999). However, this elongation stage involved two steps each 2 weeks long in 10 liters of medium with different hormone treatments. Furthermore, for higher percentage of successful *ex vitro* acclimatization Escalona *et al*. (2003) recommended that the elongation stage performed under high light intensity and for improving the shoot elongation Gonzalez *et al*. (2005) recommended addition of antibiotic

to the elongation medium. The procedure is too complex and more expensive than the *in vitro* rooting.

The elongation stage is more expensive than rooting and required that the shoots attained a minimum length of 80 mm before being transferred to acclimatization (Escalona *et al*., 1999) while rooted shoots (plantlets) as short as 30 (Be and Debergh, 2006), 40 (Sripaoraya *et al*., 2001b), 50 (Ko *et al*., 2006) and 60 mm tall (Soneji *et al*., 2002a) were successfully acclimatized. Rooting of shoots minimized the shoot length (plantlet height) requirement for acclimatization survival. At the same time rooting stage could also induce elongation of shoots and could serve dual purposes, elongation and rooting. Rooted shoots as long as 80 (Kiss *et al*., 1995) and even 165 mm long (Yabor *et al*., 2006) were obtained in one step in simple rooting medium. Plantlets height is very importants parameter, but this parameter in particularly were not used for assessment of rooting treatment and was not mentioned except in one report (Be and Debergh, 2006). The usually reported rooting parameter were rooting percentage, root number and root length and none of them are crucial for pineapple survival of acclimatization. For pineapple, the importance of rooting treatments may not its effect on induction, formation and elongation of the roots but through improving shoots hardening criteria as stomata quick response to water deficit, deposit of cuticle and wax layer on leaves surface and improving the photosynthesis capacity of the leaves. Hence, instead of the usual rooting parameters, new parameters might be more important for assessment of different *in vitro* rooting and shoot elongation treatments.

In summary, all of the so far reported *in vitro* rooting of pineapple did not serve the real purpose of *in vitro* rooting. None investigated the relation between rooting parameters and plantlet survival of acclimatization. All except Be and Debergh (2006) neglected reporting the effect of different rooting treatment on the plantlet height. Cost is the main

obstacle of micropropagation. If the explants density during rooting maintained at one shoot and the medium per culture at 20 ml per culture as it is usually done, the micropropagation definitely will always be extremely expensive. Investigation of the effect of density, size and age of shoots, medium states, strength and medium volume per culture, sucrose content and incubation period are very important for induction of rooting and very crucial for lowering the cost of micropropagation. However, most of these factors were not investigated and none was optimized.

1.4. Acclimatization of micropropagated plantlets

By the end of the rooting stage, plantlets with shoot and root system obtained but these plantlets could not withstand direct transfer from the culture vessels to outdoor conditions. The relative humidity inside the vessels is very high, the light intensity of the incubation room is very low and physiologically and morphologically the plantlets are unfit for efficient absorption and control of water loss under the low humidity and the high light intensity prevailing in the outdoor conditions. To survive, the plantlets are usually *ex vitro* hardened by transferring the plantlets to pots and trays filled with rooting substrates that could maintain enough water supply and good aeration at the root zone and by gradual decrease in relative humidity and increase in light intensity over a period of 15 to 30 days.

1.4.1. Rooting substrate types and mixing ratio

Surplus water and good aeration at the root zone during acclimatization could be maintained by selection of proper substrates and mixing ratio that provide optimum compromise between water holding capacity and substrate porosity, using of proper container size and irrigation schedule. Paz *et al.* (2000) obtained 100 % plantlets survival using sand alone under controlled light intensity and misting regime. However, enclosing the plantlets containers with polyethylene bags under polyethylene house, Aydieh *et al.*

(2000) reported that only 50 % of the plantlets survived on sand. In mix of equal part of sand and peatmoss the survival increased to 60 % while on mix of 1 part sand and 3 parts peatmoss the survival was 100 %. Wakasa (1989) obtained 50 % survival of plantlets on mix of sand, soil and vermiculite. Replacing of the vermiculite with cow dung (Khatun *et al*., 1997; Bordoloi and Sarma, 1993), peatmoss (Rahman *et al*., 2001) and compost (Akbar *et al*., 2003) increased the plantlets survival to 100, 70 and 85 % respectively. Rooting substrate made by mixing sand and cow dung plus either charcoal (Khan *et al*., 2004) or coir dust (Fernando 1986) and mixing of sand, peatmoss plus perlite (Fitchet 1993; 1990a) resulted in 100 % survival. Rahman *et al.* (2001) obtained 100 % survival in coco peat alone while survival in mix of sand, peatmoss and soil was 70 % only. Some researchers suggested autoclave sterilization of the substrates (Soneji *et al*., 2002b; Almedia *et al*., 2002; Khatun *et al*., 1997; Kiss *et al*., 1995; Bordoloi and Sarma, 1993). However, beside it would increase the cost, sterilization of substrates was not crucial for plantlet survival.

Mix of soil with one substrate as vermiculite (Omokoio *et al*., 2001), sugar cane baggasse (Escalona *et al*., 1999) and with two substrates as cow dung and coconut fibre (Sripaoraya *et al*., 2006) were used during acclimatization of pineapple. Noval *et al.* (1995) favored mix of soil plus zeolite over soil plus sheep manure, soil plus filter paper cake and soil alone while Yabor *et al.* (2006) used zeolite and filter paper cake and misting. Substrates made of perlite alone (Singh and Manual, 2000), perlite plus compost (Sripaoraya *et al.*, 2003), perlite plus peatmoss (Soneji *et al*., 2002a; Mhatre and Rao, 2002a, b) and perlite plus compost and vermiculite (Sripaoraya *et al.*, 2001a) were also used during acclimatization. Liu et al. (1989) used promix and misting under greenhouse while Bhatia and Ashwath (2002) suggested vermiculite and poting mix in temperature controlled room and mist for 2 weeks before transferring the pots to greenhouse. However, in all of these acclimatization experiments very little details were provided. The percentage

of plantlets survival was not equivocally reported and the substrates and mixing ratio were not compared with other substrates and mixing ratio. Substrates varied in cost and availability in different countries and their selections are management decision. However, the mixing ratio of any substrates should be selected based on supporting experimental results. Mix with high water holding capacity and good aeration require a compromise between proper substrate types and mixing ratio and irrigation time while water absorption improved by avoiding damaging the root system of the plantlets.

1.4.2. Relative humidity and light intensity

In vitro plantlets are physiologically and morphologically unfit for water loss control and quick response to water stress. Direct transfer to out door would result in desiccation and withering of the plantlets. Gradual decrease of relative humidity and increase of light intensity and surplus supply of water are crucial factors for successful acclimatization and survival under outdoor conditions. To ensure high relative humidity several approaches have been suggested ranging from individual enclosing of each plantlet (pot) by polyethylene bag (Rahman *et al*., 2001; Singh and Manual, 2000; Aydieh *et al*., 2000; Khatun *et al*., 1997), cover of several plantlets (trays, pots) by polyethylene sheet (Akbar *et al*., 2003), placing of plants (pots, trays) inside propagator with transparent lid and closed ventilation holes (Sripaoraya *et al*., 2003), automatic periodical misting (Yabor *et al*., 2006; Gangopadhyay *et al*., 2005; Khan *et al*., 2004; Escalona *et al*., 1999). Gradual decrease of relative humidity could be achieved by gradual removal of cover, opening of the ventilation holes of propagator and changes in misting time. Complete hardening to relative humidity could be accomplished in 15 to 21 days. Light intensity, on the other hand, controlled by shading using cloth (Ko *et al*., 2006; Almeida *et al*., 2002, Garcia *et al*., 2000) and Saran nets of various netting percent (Sripaoraya *et al*., 2006; Singh and Manual, 2000; Khatun *et al*., 1997). Gradual removal of shade or using of net of various

netting percent over a period of 75 to 90 days adapt the plant to natural light. During this period the plants maintained under normal irrigation and fertilization regimes for best growth and development and protected from full sunlight until new leaves produced with full photosynthetic ability. For best acclimatization and growth, Paz *et al.* (2000) suggested two different light intensity and misting regimes. The first 30 days, the plants kept under light intensity of 222 - 458 umol / m^2 / second accompanied with misting for 3 minutes every half hour for the first week, 2 minutes misting for the second week and 30 seconds misting for the third and fourth weeks. Then the light intensity increased to 920 umol / m^2 / second, misting stopped and replaced with daily watering for 45 days. Once the plants developed new leaves under the acclimatization conditions, reached about 100 mm in height and 5 gram in weight, usually within 75 days, it become photosynthetic full active and could be exposed to direct sun light.

1.4.3. Plantlets survival and growth

Different pineapple survival percentages were reported using different substrates and mixing ratio and it is clear that improper selection of substrate could cause a great loss of plantlets. However, whether the optimal substrates varied at different plantlets quality and under different means of relative humidity control were not investigated. Plantlets of different heights, 30 (Be and Debergh, 2006), 40 (Sripaoraya *et al*., 2001b), 50 (Ko *et al*., 2006), 60 (Soneji *et al*., 2002a), 80 (Kiss *et al*., 1995) and even 165 mm long plantlets (Yabor *et al*., 2006) were used and successfully acclimatized. However, the relation between plantlets quality as age (length of incubation period during *in vitro* rooting), root number per plantlets, root length and plantlets height and the plantlets survival percentage and growth during acclimatization were not discussed. It is possible that at certain quality, the plantlets could not survive unless certain substrates, mixing ratio and relative humidity control are used while at other quality survival could be obtain at wide range of substrate

types, mixing ratio and humidity control. For instance, misting may be obligatory for short plantlets and plantlets that were rooted for shorter incubation while 100 % survival of plantlets that were rooted for longer incubation and tall plantlets could also be obtained by enclosing the plantlets by polyethylene sheet. Similarly, all plantlets may survive at any substrate (sand alone) if the plantlets had more roots while plantlets with fewer roots survived only if certain substrate (peatmoss or perlite) alone or at specific mixing ratio were used. It is very important to investigate the effect of these three factors, substrates, relative humidity control means and plantlets quality on the plantlets survival and the acclimatization procedures adjusted accordingly and judged by cost comparison. Glasshouse, automatic misting and high light intensity supplement and using of perlite and vermiculite may not be crucial for survival of plantlets with certain quality and could be just an extra added cost that could be saved. Unfortunately, the relation between plantlets quality and survival under different substrates type, mixing ratio and means of humidity control were not investigated. Arbitrary adoption of any acclimatization procedure is just either taking a risk of losing the plantlets or unjustified cost.

Generally, survival of all pineapple plantlets (100 %) was obtained under different acclimatization procedures. Different procedure may fit one geographical area than others. However, the growth rate was usually slow and 6 to 12 months might be needed before the plantlets reached the growth specifications of good planting material for field planting. Inoculation of mycorrhiza was suggested to increase plantlets survival (Jaizme and Azoon, 1991), enhancement of growth (Noval *et al*., 1995) and to increase tolerance to *Phytophthora cinnamomi* (Guillemin *et al*., 1994). However, beside strain specificity, dependence of infestation on substrate properties and mineral contents and cost of inoculation, the results was not always consistent. Using larger pots and under long days condition, Folliot and Marshal. (1991) reported that plantlets weighted 2 grams at the

beginning of acclimatization could reach 106 grams and attain the salable quality in 4.5 months. Generally, the plantlets should be kept protected from direct sunlight until it forms new phytosynthetic active leaves and then either left inside the greenhouse or transfer to open nursery for 2 to 6 months. Once the plantlets reached 150 mm in length, 150 grams in weight (Almeida *et al*., 2002; Folliot and Marshal, 1991) and developed about 30 leaves it can be sold as propagules ready to be planted in farms. High plantlets survival, growth rate and fast development might be better achieved through timing of acclimatization to months of long days according to the geographic location of the country in which the project are conducted, developing of an *in vitro* hardening procedures and adopting of *in vitro* induction but *ex vitro* development of roots.

1.5. Cost and commercial production

The reported multiplication rate and expected total after several months and year did indicate potential for mass production of pineapple propagules. But close look on the data provided in these literatures indicated that adoption of any of these suggested protocols for commercial pineapple micropropagation is very unlikely. Many details were missed and in all studies the cost neither per shoot, per subculture, at each stage nor even as roughly total was estimated. None of the micropropagation stages starting from decontamination of primary explants and establishment up to multiplication and rooting has been optimized. Many methods of sterilization were used but none reproducible. It is very obvious that unless large number of primary explants were successfully decontaminated and established, lag period of 8 to 12 months would elapse before enough shoots become available to start commercial production using conventional micropropagation system (Dewald *et al*., 1988) or bioreactor system (Firoozabady and Gutterson, 2003). Long lag time is a serious drawback and important cost factor.

Although Escalona *et al*. (1999) and Firoozabady and Gutterson (2003) said respectively that the use of bioreactors could reduce the cost by 20 and 35 %, it is very difficult to comprehend claim of cost reduction without providing the final figure of total cost and cost per unit of production and itemization of the various cost factors. The bioreactor system reduced the culture containers and shelves space compared to conventional. However, taking in consideration the cost of the 30 liters of medium (full strength MS) that were used per one bioreactor cycle, cost of apparatus and maintenance of the bioreactor system, the total shoot produced per bioreactor cycle (3000 shoots) did not support the claim of low cost per single shoot. Furthermore, converting the 13 shoots per explant obtained in 20 ml of static liquid (Teixiera *et al*., 2006) and 65 shoots per two explants obtained in 50 ml of medium agitated at 100 rpm (Soneji *et al*., 2002a) to shoot production per liter of medium indicated that 650 shoots could be produced per liter of medium. The number of flasks used for one liter is 50 and 20 respectively and the shoots produced per liter of medium (650 shoots) were three times higher than that obtained in bioreactor system (200 shoots per liter) with two 10-L vessels (Escalona *et al*., 1999). Similarly, the 10 shoots per explant obtained in 50 ml of solid medium (Sripaoraya *et al*., 2003) indicated that 200 shoots could be produced per liter. The advantages of bioreactor may not as had been claimed.

Most importantly, in all reported studies of pineapple micropropagation the effect of many factors as explant size and density, medium strength, sucrose concentration and medium volume per culture were totally ignored and the salt formulation was only tentatively studied. If such factors investigated, it is possible that a new protocol more cost effective than the available ones could be developed. Amount of medium and number of vessels and consequently the shelving space during any multiplication cycle is strongly related to explant density and volume of medium per vessel. The cost of medium depends

on its type, strength, sucrose content and volume. To minimize the cost, the effect of these factors needs to be studied and optimized. Commercial application not only required a valid estimation of total or average of shoot per explant after several cycles but also the total and rate of shoot formation at each and over consecutive subcultures, timetable and schedule of operations, amount of medium, vessels and shelving space required for each multiplication cycle and during rooting stage. Otherwise, commerciality of the protocol is questionable.

1.6. Fidelity of micropropagated pineapple

Frequency of spineless and spine variants among spiny and spineless cultivar of pineapple is the simplest indicator of variations and very reliable estimator of fidelity of pineapple. Spiny and spineless is the most common and most frequently reported variation compared to any of the other morphological aspects. In fact, up to maturity, spiny and spineless was the only different observed among *in vitro* obtained Smooth cayenne (Firoozabady *et al*., 2006, Firoozabady and Moy, 2004; Firoozabady and Gutterson, 2003), Queen (Escalona *et al*., 1999) and Red Spanish (Liu *et al*., 1989). Variation in leaf spine can be seen at early stage of multiplication, rooting, acclimatization and nursery. Although not all plantlets which showed spiny variation would definitely showed variation in flowering and fruit quality (Firoozabady and Moy, 2004; Firoozabady and Gutterson, 2003), all plants which showed fruit and flowering variation were spiny variants (Smith *et al*., 2002; Das and Bhowmick, 1997). Every single shoot and plant can be screened by naked eye for presence and absence of spine while using of isozymes and DNA profile represents a sample within population and used as indicator of probable percentage of variation among large population. Furthermore, using of isoenzyme and DNA did not indicate variation percentage higher than that detected by spiny and spineless plantlet

frequency. The variation among none transformed shoots detected by isozymes was 0.67 % and that by DNA was 7.5 % (Feuser *et al.*, 2003) and that by spiny was also 6 % (Firoozabady *et al*., 2006; Firoozabady and Gutterson, 2003, Soneji *et al*., 2002 a). Soneji *et al.* (2002c) confirmed that spiny and spineless Queen pineapple had different DNA banding pattern.

Variations among micropropagated pineapple do not pose threat to commercial production of pineapple planting materials. Firstly, several researchers reported production of plantlets identical to mother plants and in cases variation occurred, the frequency of variation was within a manageable range (3 to 6 %) and equal to variations percentage that usually observed on plants obtained using conventional propagation methods. Secondly, the variations can be seen during the flask stage, multiplication and rooting and discarded and would be easily rogue out at acclimatization and in nursery. Thirdly, most of the variations occur in plant foliage particularly the leaves spine with none to a very low percent of variation in flowering time and fruit chemical and morphological characters. Fourthly, the variations which appeared late at maturity in flowering and fruits are usually associated with morphological character as spiny and spineless leaves that can be seen during flask stage. Fifthly, the percentage of variation is closely related to type of explants, mode of regeneration and multiplication systems could be avoided or at least minimized to very low percentage by proper management.

Appearing of spiny leaves in spineless variety such as Smooth cayenne and spineless leaves in spiny variety as Queen has been reported by almost all researchers and it is the most frequently observed type of variation during flask stage, acclimatization and in field. The frequency of variation in spiny and spineless leaves ranged from 3 to 7 % (Firoozabady and Gutterson, 2003; Escalona *et al*., 1999; Soneji *et al*., 2002a). Other morphological variations such as Albino, white streaks (Fitchet 1990b; Mathew and

Rangan, 1981; Firoozabady and Gutterson, 2003; Soneji *et al*., 2002a), anthocyanin streaks and elongated internodes (Soneji *et al*., 2002a) and condense leaves (Ko *et al*., 2006; Fitchet 1990b; Wakasa, 1989) were observed during multiplication stage (flask stage) at frequency lower than 2 %. Anthocyanin streak (Soneji *et al.,* 2002a), white strip (Sripaoraya *et al*., 2003) and high wax contents (Garcia *et al*., 2000) were observed also during acclimatization at frequency of about 7, 4 and 1.5 % respectively. Although the frequency in spiny was as high as 6 % (Firoozabady and Gutterson, 2003. Sripaoraya *et al*., 2003), the flowering and fruiting was identical to that of the mother plants. Similarly, Das and Bhowmick (1997) observed 4 % variation in leaf morphology while the variation in fruit was less than 0.1 %. Singh and Manual (2000), on the other hand, reported that micropropagated pineapple had slower growth rate and flower earlier and Smith *et al*. (2002) and Das and Bhowmick (1997) observed fruit of different shape. Excessive slipping, dwarfing and small fruit appeared during the first crop, but disappeared during ratoon crop (Ko *et al*., 2006). Liu *et al.* (1989) reported 14 % reversion of spineless Red spanish pineapple to spiny and 32 % of spiny to spineless. Reversion of spine and spineless was also observed by Garcia *et al.* (2000). However, Wakasa (1989) reported that up to maturity the variation in spiny and other characters was of permanent nature.

Materials and Methods

2.1. Establishment of pineapple shoots tip culture

2.1.1. Pineapple shoots tip sterilization

Crowns of Smooth cayenne were collected from public market and shoots and suckers of Moris pineapple from field growing Moris. The collected plant materials were left to dry for one day and then defoliated and washed under running tap water for a half hour. The terminal meristem was trimmed to 1.0 cm^3 and soaked in solutions of different sterilants at different concentrations and different times as follow:

1- Clorox (25 %) for 25 minutes

2- $HgCl_2$ (0.1 %) for 1 minute; Clorox (10 %) for 15 minutes

3- Clorox (25 %) for 25 minutes; $HgCl_2$ (0.1 %) for 1 minute

4- Clorox (10 %) for 15 minutes

5- Clorox (25 %) for 25 minutes; Clorox (10 %) for 15 minutes

6- Clorox (25 %) for 25 minutes; $HgCl_2$ (0.1 %) for 1 minute; Clorox (10 %) 15 minutes

7- $HgCl_2$ (0.1 %) for 5 minutes

8- Ethanol (50 %) for 5 minutes; Clorox (10 %) for 15 minutes

Under laminar flow air cabinet, the sterilants were decanted and the explants rinsed in sterilized distilled water for three times, placed in autoclaved petri dish or aluminum foil, trimmed to 0.5 cm^3 and cultured individually in glass jars (15 x 5 cm.) containing 20 ml of agar solidified (7.0 g/l) full strength MS medium (Murashige and Skoog, 1962) enriched with sucrose at 30 g/l. The cultures were kept in a culture room under a photoperiod of 16 hours of light and constant temperature 24 ^{0} C for 45 to 60 days. The number of uncontaminated (growing and nongrowing) and contaminated but growing explants was

recorded every week for 45 days. The sterilization experiment repeated two times using at each time 4 to 9 explants per each treatment.

2.1.2. Establishment of shoot tip

One liter of full strength MS medium was prepared from stock solutions enriched with sucrose at 30 g/l and divided into 4 glass jars (180 ml each). The medium in each jar was enriched with 6-benzyleaminopurine (BAP) at four different concentrations (2.25, 3.25, 4.25 and 5.25 mg/l, adjusted to pH 5.7 and dispensed into 9 jars (20 ml each). 0.14 grams of agar was added to each jar and the media were sterilized by autoclaving at 121^0 C and 1.5 kg/ cm^2 for 25 minutes. Smooth cayenne shoot tips were sterilized in Clorox at 25 % for 25 minutes, rinsed in sterilized distilled water, trimmed to 0.5 cm^3 and cultured individually one shoot tip per each jar. The cultures were kept in a culture room under a photoperiod of 16 hours of light and constant temperature 24 ^{0} C. The cultures were observed weekly and the percentage of clean growing, clean nongrowing and growing but contaminated explants were recorded. The number of shoots per culture was counted after 60 days of incubation. The experiment was repeated several times to get at least a total of 9 uncontaminated growing, uncontaminated but nongrowing and growing but contaminated explants per each hormone treatment. Data were arranged in table of three replicates and subjected to analysis of variance and Duncan Multiple Range Test at p 0.05 using SPSS statistical package No.11.

2.2. Shoot multiplication

2.2.1. BAP singly applied and in combination with IAA

Four liter full strength MS medium were prepared from stock solutions, enriched with sucrose at 30 g/l and divided into 20 glass jars (180 ml each). Each jar was enriched with one of the following hormone treatments: BAP alone at 2.25, 3.25, 4.25 and 5.25 mg/l and

in combination with IAA at 0.75, 1.75, 2.75 and 3.75 mg/l. The pH was adjusted to 5.7 and the content of each jar was divided equally into 9 glass jars (20 ml each) and 0.14 grams of agar were added to each jar. The jars were closed with autoclavable plastic lid and the media were autoclaved for 25 minutes at 121^0 C and 1.5 kg /cm^2. Shoots from Smooth cayenne stock cultures were cultured individually one shoot per jar and the cultures were incubation under constant temperature at 25^0 C and 16 hours of light. After 60 days of incubation, the multiple shoots complex were picked out of the cultures (jars) and separated into individual shoots for counting shoots and measuring shoot length. The data were arranged in table of three replicates, each replicate average of three jars, and subjected to analysis of variance, regression and correlation analysis and Duncan Multiple Range Test at p 0.05 using SAS statistical package 6.12.

2.2.2. Hormones and incubation periods

Nine liters of full strength MS medium was prepared from stock solutions using 7 beakers each contained 1260 ml of medium and marked 1 to 7. The medium of each beaker was enriched with sucrose at 30 g/l and one of the following hormone treatments:

BAP at 2.25 mg/l alone and in combination with IAA at 0.175, 0.75 and 1.75 mg/l

BAP at 2.25 mg/l plus IAA at 1.75 mg/l plus GA_3 at 1.0 mg/l

BAP at 3.25 mg/l in combination with IAA at 1.75 and at 3.75 mg/l

The media were adjusted to pH 5.7 and the content of each beaker was transferrred into 63 glass jars (20 x5 cm) each with 20 ml and marked with the same beaker number. Agar (0.14 g) was added to each jar and the jars were closed with autoclavable plastic lid and the media were autoclaved at 121^0 C and 1.5 kg /cm^2 for 25 minutes. Shoots from Smooth cayenne stock cultures were cultured, one shoot per jar and the cultures were incubated under constant temperature of 25^0 C and 16 hours of light (30 mmol m^{-2} s^{-1}) provided by cool white florescent lamps. After each 15 days for a period of 105 days 9 jars from each

hormone treatment were taken out of the incubation room and the multiple shoots complex of each jar was picked and weighted (total weight per explant) and then separated into individual shoots for counting the number and measuring the length of each shoot. For each jar, the total shoots length was calculated by summation of shoots length of all shoots and average shoot length computed by dividing the total shoot length over the shoot number. After 105 days, four tables for each of the 49 combinations (7 incubation periods and 7 hormone treatments) each with data recorded from 9 jars were constructed, one for total weight, one for total shoot number, one for total shoot length and one for average shoot length per jar (explant). Each treatment consisted of 9 jars (cultures) and data of each three jars were averaged to establish three replicates tables for each of the parameter. These tables were converted to tables of biweekly increase in shoot number, total shoot length and total weight for each of the different hormonal treatments by subtraction of the values obtained at replicate I, II and III of one incubation period from that of the replicate I, II and III of the previous incubation period. Before subjected to ANOVA analysis and Duncan Multiple Range Test for means separation, 0.1 were added to data of shoot number and 0.01 to data of total shoot length and weight.

2.2.3. Hormones, incubations and subcultures

Each time one or two days before data collection from the shoot incubated for 30, 45, 60 and 75 days in the above experiment (2.2.2), 1300 ml of full strength MS medium was prepared from stock solutions, enriched with sucrose at 30 g/l, divided into 7 jars (180 ml each) and enriched with one of the 7 hormone treatments. The medium pH was adjusted to 5.7 and the medium was divided into 9 jars to each 0.14 grams of agar was added and sterilized for 25 minutes at 121^0 C and 1.5 kg/ cm^2 . After 30 days of incubation, one or two of the 9 jars from each of the seven hormone treatments were taken out of the culture room, the multiple shoots complex of each jar was picked out and weighted under laminar

using sterilized aluminum foil and two digit balance, placed on sterilized petri dish and separated into individual shoots. The number and the length of the shoots were recorded and 9 shoots were subcultured individually, one shoot per jar containing the same hormone treatment. The multiple shoots complex of the other jars was weighted and the shoot number counted and length measured under room condition. From the 7 hormone treatments and 9 jars data recorded after 30 days of incubation, four tables were constructed for total weight, shoot formation rate per explant (jar), total and average shoot length. Every three of the nine cultures were considered as replicate and the tables converted to tables with three replicates (each replicate was average of 3 jars) and considered as data of shoot formation per explant of the first subculture of the 30 days long incubation period.

The same procedures of subculturing, data collection and tables construction used for the different hormone treatments during the first subculture of the 30 days incubation were used also for the culture (jars) incubated for 45, 60 and 75 days to establish the data tables of the first subculture of the 45, 60 and 75 days incubation period. Data tables for the second, third and fourth subculture of the 30, 45, 60 and 75 days incubation periods were established following the same procedure used during the first subculture. After 4 subcultures, each of the tables obtained after each subculture for the same parameter (total weight, shoot formation, total and average shoot length) were combined in one table with three replicates for the 112 combinations (4 incubations, 7 hormone treatments and 4 subcultures). The data of shoot formation rate and average shoot length per explants were subjected to two ways analysis of variance as function of hormone treatments and incubation periods overall subcultures using SPSS statistical package No. 11 and the significant of means were tested by Duncan Multiple Range Test at $p \leq 0.05$.

Total shoots production after consecutive subcultures were established from the table of shoot formation per explant. The shoot formation per explant of the the three replicates of the first subculture was averaged and considerd as the total shoots of the first subculture. The total shoots of the first subculture multiplied by the shoot formation per explant obtained on each replicate of the second subculture and the total of three replicates averaged to obtain the total shoots after two consecutive subcultures. The total shoots obtained after two subcultures multiplied by the shoot formation per explant obtained at each replicate of the third subculture and the total shoots of three replicates averaged to obtain the total shoots after three subcultures. The total shoots after three subcultures multiplied by the shoot formation per explant obtained at each replicate of the fourth subculture and the total of three replicates averaged to obtain the total shoots after four subcultures. A table with three replicates for total shoots after 4 consecutive subcultures was established for the 7 hormone treatments at 4 incubation periods (30, 45, 60 and 75 days). The total shoot data were transformed using log 10 and subjected to two ways analysis of variance to test the effect of different hormone treatments and incubation periods and the effect of incubation periods and subcultures at one fixed hormone treatment (BAP 3.25 plus IAA 1.75 mg/l) using SPSS statistical package No. 11. Significant of means was tested by Duncan Multiple Range Test at $p \leq 0.05$

The percentage of shoot formation of each subculture during the first 30 days of a 75 days long incubation was computed by dividing the average shoots obtained after 30 days by that obtained after 75 days and the rate per week by dividing by 4.29 (number of weeks per month). The percentage of shoots formed during the period between 30 to 45, 45 to 60 and 60 to75 days of incubation was computed by subtracting the average obtained at shorter incubation from the next longer incubation and divided by the shoot average obtained after 75 days of incubation while the rate of shoot formation per week during the

15 days interval of incubation was obtained dividing by 2 (number of weeks). The shoot formation rate per week at a 30, 45, 60 and 75 days long incubation was obtained by dividing the average number of shoots obtained at that incubation by 4.29, 6.4, 8.6 and 10.7 (number of weeks), respectively.

2.2.4. BAP single application

Three liters of full strength MS medium was prepared from stock solutions, enriched with sucrose at 30 mg/l and divided into 9 jars (each 180 ml) marked with No 1 to 16. No hormone was added to jar No 1 and BAP at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00, 3.25, 3.50 and 3.75 mg/l were added to the jars No. 2 to 16 respectively and the pH was adjusted to 5.7. The content of each jar was dispensed into 9 jars (20 ml each), 0.14 grams of agar was added to each jar and the media were autoclaved at 121^0 C and 1.5 kg /cm^2 for 25 minutes. Shoots from Smooth cayenne stock cultures were cultured one shoot per jar and the cultures were incubated under constant temperature of 25 ^{0} C and 16 hours of light provided by fluorescent lamp. After 60 days, the cultures were taken out of the incubation room and the multiple shoots complex were picked up, weighted and separated into individual shoot for counting number of shoots and measuring shoot length. The weight of multiple shoot per each culture was considered as total weight per explant. The length of shoots were summed and considered as total length per explant. Average weight and length per explant were computed by dividing total weight and total length by the number of shoot per explant. Each treatment consisted of nine cultures and tables with nine figures were established for each parameter. The data were subjected to analysis of variance, regression and correlation analysis and Duncan Multiple Range Test for mean separation at $p < 0.05$ using SPSS static package No.11.

2.2.5. Explants density and explants manipulation

MS medium (1.3 L) was prepared from stock solutions, supplemented with sucrose at 30 g/l and BAP at 2.25 mg/l and adjusted to pH 5.7. The medium was dispensed into 63 glass jars (5x 15 cm) each received 20 ml of medium and 0.14 agar was added to each jar. The jars were closed with autoclaveable plastic lid autoclaved at 121 ^{0} C and 1.5 kg / cm^2 for 25 minutes. Smooth cayenne stock cultures were used as source of explants. The multiple shoots complexs were either separated into individual shoots or into clusters of 2, 3 and 4 shoots. The separated shoots were cultured at density of 1, 2, 3 and 4 per culture and the shoot clusters (connected shoots) were cultured at density of one cluster but of different size (2, 3 and 4 shoots per cluster). Each treatment of different density and cluster size consisted of 9 cultures. All cultures incubated under constant temperature of 25^0 C and 16 hours of light provided by florescent lamps. After 60 days of incubation, the multiple shoots developed from each explant was individually removed from the cultures, weighted, separated into individual shoots for counting the number and measuring the length of shoots. Tables for total shoots per culture for all of the 9 cultures (jars) per treatment was constructed from sum of the shoots produced from all explants per culture (jar) and table for shoot formation per explant was established by dividing the total shoots per culture by the explant density per culture (number of separated shoots and number of shoots per cluster). Table for average length of shoot was established by dividing the summation of the length of all shoots per culture (jar) by the total shoots per culture (jar). The tables were converted to three replicates tables by using the average of each three of the nine cultures. Total shoot production per one liter of medium, on the other hand, was estimated by multiplication of each replicate of the total shoots data table by 50. Data were subjected to ANOVA and means separated by Duncan Multiple Range test at $p \leq 0.05$ using SSPS statistical package No. 11.

2.2.6. Hormone types, concentrations and subcultures

Four liters of full strength MS medium were prepared from MS powder, supplemented with sucrose at 30 g/l and divided into 164 glass jars (15 x 5 cm.) each received 20 ml of the medium. The jars were divided into five groups. The first group consisted of 4 jars, each jar was marked with number 1, the second, third, fourth and fifth group each consisted of 40 jars and each 4 jars of the second marked with number 2 to 11 and each 4 jars of the third, fourth and fifth groups were marked with number 12 to 21; 22 to 31 and 32 to 41 respectively. No hormone were added to the first group of jars and BAP, KN, NAA and IAA each at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mg/l were added to the second (2 to 11), third (12 to 21), fourth (22 to 31) and the fifth (32 to 41) group of jars respectively. The medium of each jar was adjusted to pH 5.7 and 0.14 grams of agar was added to obtain a 7 g/l agar solidified medium. The jars were closed with autoclaveable plastic lid and the media were autoclaved at 121 ^{0} C and 1.5 kg/cm^{2} for 25 minutes. Shoots from Moris pineapple stock cultures were cultured at density of one shoot per jar and the cultures were incubated at constant temperature of 25 ^{0} C and 16 hours of light. After 2 months, fresh medium of each combination of hormone types and concentrations were prepared as above but the jars of the first group increased to 9 jars and that of the second, third, fourth and fifth groups increased to 90 jars. The cultures were taken out of culture room, the multiple-shoots complex of one to two jars of each combination of hormone types and concentrations were picked out of the jars one after another under laminar floor and placed in autoclaved petri dish under which a squared paper was placed to facilitate measuring of shoot length. The multiple shoots complex separated into individual shoots and the shoot number and length were recorded and 9 shoots were subcultured into the newly prepared media one shoot per jar containing the same hormone treatment the shoots were growing in during the first subculture. The multiple shoot complex of the other jars

(cultures) was picked out and separated into individual shoot for counting the shoot number and measuring the length of shoots per complex under room condition (not laminar). The data were recorded as data of the first subculture of the different combinations of hormone types and concentrations. The cultures incubated under the same incubation conditions and after two months the same procedures used during the first subculture of preparing fresh media of different hormone treatment, 9 jars of the first group and 90 jars of the others, recording the data of the second subculture and subculturing the shoots into the newly prepared media on jars containing the same hormone treatment the shoots were growing in during the second subculture was repeated as done for the first subculture. After two months the data of the third subculture were collected in similar way.

Shoot formation rate and average shoot length per explant and total shoots over consecutive subculture were calculated as done before in section (2.2.3) and subjected to three ways analysis of variance as function of hormone types, concentrations and subcultures using SAS statistical package No 6.12 Significant of means were tested by Duncan Multiple Range Test at $p \leq 0.05$.

2.2.7. Sucrose, pH and medium states

Three liters of full strength MS medium were prepared from stock solutions. A 6.0 ml from BAP stock solution (1 mg/ ml) were added to the medium to give a concentration of 2.0 mg/l of BAP per liter. The medium divided into 4 beakers (750 ml each) marked A, B, C and D and 7.5, 15.0, 22.5 and 30.0 grams of sucrose were added to each beaker respectively to give a sucrose enrichment of 10, 20, 30 and 40 g/l. The content of each beaker was divided into another 4 beakers marked with same beaker letter and numbered 1 to 4 and the medium pH of the beakers 1, 2, 3 and 4 was adjusted to pH 5.0, 5.7, 6.0 and 6.5 respectively. The content of each beaker of the same sucrose pH combination was divided into 9 glass jars **(**20 x 5 cm**)** each received 20 ml and each three jars marked with

same number of the beaker plus S, E and L. Agar at 0.14 and 0.7 grams was respectively added to each jar marked with S and E letter to give medium solidification of 7.0 and 3.5 g/l and no agar was added to jars with L letter. The jars were closed by autoclaveable plastic lids and the medium autoclaved at 121 ^{O} C and 1.5 kg / cm ^{2} for 25 minutes and kept in a culture room. One shoot from Moris stock cultures were cultured per each jar and the cultures incubated under constant temperature of 25 ^{O} C and 16 hours of light provided by cool white florescent lamps. After 2 months of incubation, the cultures were taken out for data collection. The multiple shoots complex were picked out and separated into individual shoots for counting the number and measuring length shoot per the multiple shoots complex. Each jar was considered as a replicate and the data subjected to ANOVA analysis and significant of means was tested by Duncan Multiple Range Test at $p \leq 0.05$ using SPSS statistical package No. 11.

2.2.8. Hormone types and concentrations in liquid medium

The different hormone types and concentrations that were tested in solid medium using high sucrose (30 g/l) and large medium volume per culture (20 ml) and one shoot per glass jar (experiment 2.2.7) were retested using liquid medium, lower sucrose concentration (20 g/l) lower pH (pH5.0) and less medium per culture (10 ml), higher density (two shoots per culture) and culture tubes instead of glass jars. Thirty ml of each combination of hormone types and concentrations was prepared and in same way (experiment 2.2.7) and autoclaved using glass jar. The content of each glass jar (30 ml) was dispensed under laminar into 3 sterilized culture tubes marked with same mark of the glass jar (10 ml per culture) using sterilized syringe. Shoots from Moris stock cultures were cultured at density of two shoots per culture tubes and the cultures incubated under constant temperature of 25 ^{0} C and 16 hours of light provided by cool white florescent lamp. After two months of incubation, cultures were taken out of culture room, the multiple shoots removed under laminar and

placed in sterilized petri dish and separated into individual shoots and the shoot number and length were recorded. The total shoots per culture divided by two (explants density) and the sum of the shoots length divided by total number of shoots to get the shoot formation rate and average length of shoot per explant. Each culture (culture tube) was considered as a replicate and the data subjected to two ways analysis (hormones and concentration). Analysis of variance and significant of the mean of the different treatments were screened by Duncan Multiple Range Test at $p \leq 0.05$ using SPSS statistical package No. 11.

2.2.9. Explants density and size

Full strength MS medium (800 ml) was prepared from MS powder, enriched with sucrose at 20 g/l and BAP at 2.0 mg/l and adjusted to pH 5.0. The medium was dispensed into 2-liters glass bottle and autoclaved for 20 minutes at 121^0 C and 1.5 kg/ cm^2. Under laminar, the medium was dispensed into 75 culture tubes at 10 ml per tube using sterilized syringe. Each three culture tubes were marked with No 1 to 25. Multiple shoots complex from Moris stock cultures were placed on sterilized petri dish and separated into individual shoots under laminar floor. Shoots arranged in groups of different length (5, 10, 15, 20 and 25 mm long) and the 5 mm long shoots were cultured at density of 1, 2, 3, 4 and 5 shoots in the culture tubes No 1, 2, 3, 4 and 5. The 10, 15, 20 and 25 mm long shoots were cultured at density of 1, 2, 3, 4 and 5 shoots on the culture tubes No. 6 to 10, 11 to 15, 16 to 20 and 21 to 25 respectively. Three culture tubes (each considered as a replicate) were used for each combination of explant size and density and the cultures incubated under constant temperature of 25 ^{O} C and 16 hours of light for 60 days. After two months of incubation, the culture tubes were taken out of the incubation room and the multiple shoot complex of each culture tube were removed and separated into individual shoots using scalpel and tweezer. The number and length of shoots per each multiple shoots complex

per each culture tube was measured and recorded. Each culture tube of the same explants density and explants size was considered as a replicate and the recorded data of shoot number per each culture were rearranged into 7 different tables each with three replicates. One table was for total shoots per culture (irrespective of shoot length). The total shoots per each culture tube were assorted into different shoot lengths rank (< 5; 6-10; 11-15; 16-20; 21-25; > 26 mm) and 6 tables were constructed for the shoot number per each shoot length rank. To avoid the zero values for shoot number of some of the shoot length ranks, the data were transformed by adding 0.01 to the data of all the 6 tables. The table of total shoots (irrespective of shoot length) and tables of total shoots of each shoot length rank per culture were converted to tables of shoot formation per explant by dividing by the explants density per culture and to tables of total shoots production per one liter of medium by multiplying by 100. The shoot length data arranged in one table of average shoot length per culture established by dividing the sum of length of all shoots per culture by the total shoots per culture (irrespective of their length) and converted to average shoot length per explant by dividing by the explant density per culture. The data of each parameter analyzed by two ways ANOVA and the treatments means separated by Duncan Multiple Range Test at $p \leq 0.05$ using SPSS statistical package No.11.

2.2.10. Medium volume and explants density per culture

One liter of full strength MS medium was prepared from MS powder and supplemented with 20 grams of sucrose and 2.0 mg of 6-benzylaminopurine (BAP). The medium pH adjusted to 5.0, transferred to 2-liter glass bottle and autoclaved at 121 ^{O} C and 1.5 kg/cm ^{2} for 25 minutes. The medium dispensed under laminar into 120 plastic culture tubes (40x 100 mm). Each 12 tubes marked with the same symbol (V1 to V10) and received equal amount of medium, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 ml using sterilized syringe. In each three of the 12 culture tubes that contain equal volume of medium, shoots

from stock cultures of Moris pineapple were cultured at density of 1, 2, 3 and 4 shoots per culture and number 1 to 4 was added to each volume symbol of the culture containing 1, 2, 3 and 4 explants per culture (V11, V12, V13, to V101, V102, V103, V104). The cultures were placed in culture room under constant temperatrure of 25 ^{O} C and 16 hours of light provided by florescent lamps in three different shelves where each shelf represent one replicate of each combination of medium volume and explant density. After 75 days of incubation, the cultures removed from the culture room and the multiple shoots complex of each explant of the same culture tube were picked out individually, placed over squared paper and the shoots of each complex separated using scalpel and tweezer for counting the number and measuring the length of the shoots. The number of all shoots per each culture tube were summed and divided by the number of explants per tube to compute the average shoot formation per single explant and the length of all shoots per each tube were summed and divided by total number of shoots to compute the average length of shoot. The average shoot formation per culture multiplied by (1000 / the medium volume per culture) to compute the expected total of shoots per one liter of medium. Each tube was considered as a replicate. A table of three replicates was established for each parameter (shoot formation and shoot length per explant and total shoot per one liter) and used for statistical analysis. Analysis of variance and significant of treatment means were conducted using SPSS statistical package 11.0 and Duncan Multiple Range Test at $p \leq 0.05$.

2.2.11. Incubation periods, medium volume and explants density

Full strength MS medium (1200 ml) was prepared in 2-liters glass bottle from MS powder, enriched with sucrose at 20 g/l and BAP at 2.0 mg/l, adjusted to pH 5.0, sterilized in autoclave at 121 ^{0}C and 1.5 kg /cm^{2} for 25 minutes and dispensed under laminar into 144 culture tubes. Each 36 cultures were marked with A, B, C and D and received 3, 6, 9 and 12 ml respectively. To each three culture tubes of same mark, number 1, 2 and 3 were

added (A1, A2, …….D3). One, two and three shoots from Moris stock cultures were placed in each culture tube marked with number 1, 2 and 3 respectively. The cultures were placed in culture room and kept under constant temperature 25 ^{0}C and 16 hour of light provided by cool white fluorescent lamps. After, 30, 45, 60 and 75 days, 9 cultures having same mark (A1, A2, A3 up to D1, D2, D3) were randomly picked and used for data collection. The multiple shoots complex of each explant per each culture tube picked out and placed over squared paper. The shoot complex eparated into individual shoots for counting the number and measuring the length of shoots per culture tube. Shoot formation per explant were computed by dividing the total number of shoot per each culture tube by the number of explants per culture tube and the average shoot length by summation of all shoots length and dividing by total number of shoots per culture tube. Expected total per liter of medium were calculated by multiplication of total shoots obtained per culture tube by 1000 and dividing the result by the medium volume which was dispensed in that culture tube. Data subjected to analysis of variance and the significant of means tested by Duncan Multiple Range Test at 0.05 using SPSS statistical package No 11.

2.2.12. Medium strength, pH and medium volume per culture

Double strength MS medium was prepared by dissolving of 5.89 grams of MS powder in 750 ml of distilled water. The medium were then divided in 4 beakers each received 75, 150, 225 and 300 ml, marked H (half strength), S (full strength), O (one and half strength) and D (double strength) and the medium strength of each beaker was adjusted respectively to half (0.5x), full (1.0x), one and half (1.5x) and double (2.0x) strength by completing to a final volume of 300 ml using distilled water. All media supplemented with sucrose at 20 g/ l and BAP at 2.0 mg/ l. Medium of same strength were divided equally into three glass jars (100 ml each) marked with same letter plus number 1, 2 and 3 (H1, H2, H3; S1, S2,…..D3) and the medium pH of the jars marked with number 1, 2 and 3 adjusted to 5.0, 5.5 and 6.5

respectively. The jars were closed with autoclavable plastic lids and the media sterilized by autoclaving at 121^0 C and 1.5 kg/ cm^2 for 20 minutes. Media of each jar was dispensed under laminar into 18 culture tubes each three tubes received 3, 4, 6, 7 and 8 ml. and each three culture tubes of equal medium volume marked with the same jar mark plus No 1 to 72. For the jar marked H1 each three tubes of equal medium volume (3, 4, 5, 6, 7 and 8 ml) were numbered 1 to 6 respectively. For jar marked H2, the tubes were numbered 7 to 12 and for jar marked H3 the culture tubes numbered 13 to 18 respectively. The same numbering used for jars marked S1 to 3 (19 to 36), O1 to 3 (37- 54) and D1 to 3 (55 to 72) respectively. Three separated shoots from Moris stock cultures were placed in each culture tube. The cultures were maintained under constant temperature of 25^0 C and 16 hour of light provided by fluorescent lamps. Each tube was considered as replicate with three replicates per treatment. After two months, the cultures was taken out of the culture room, the multiple shoots complex of each explant was picked out of the culture tubes and separated into individual shoots for counting the number and measuring the length of shoots. Total number of shoots per each culture was either divided by three or multiplied number of cultures per one liter of medium (1000 /medium volume per culture) to establish two tables each with three replicates for average shoot formation per explant and total shoots per one liter of medium respectively. Shoot length of all shoots of the same culture tube were summed and divided by the total number of shoots to establish table with three replicates for the average length of shoot. The data subjected to analysis of variance and mean separation by Duncan Multiple Range Test at $p \leq 0.05$ using SAS statistical package 6.12.

2.2.13. Explants density, medium volume per culture and subcultures

Half liter of full strength MS medium was prepared from MS powder in 1-liter glass bottle, supplemented with sucrose at 20 g/l and BAP at 2.0 mg/l and the pH to 5.0, The medium sterilized by autoclaving at 121 ^{0} C and 1.5 kg/ cm^{2} for 20 minutes. The sterilized medium dispensed under laminar into 75 culture tubes each 25 tubes were marked A, B and C and received 4, 6 and 8 ml respectively. Number 1, 2, 3 and 4 was written on each 12, 6, 4 and 3 of the cultures tubes marked with A, B and C. Moris stock cultures were used as source of explants and 1, 2, 3 and 4 explants were added to all culture tubes marked with No 1, 2, 3 and 4 respectively. The cultures incubated under constant temperature 25 ^{0}C and 16 hours of light provided by cool white florescence lamps. After 60 days of incubation, fresh media was prepared as above, cultures were taken of incubation room, and under laminar floor the multiple shoots complex were picked and separated into individual shoots for counting number (total shoots per culture) and measuring length of the shoots per culture.12 shoots from each combination of medium volume and explants density were subcultured on the same medium volumes and explants density combination on the newly prepared media. The data recorded called the first subculture data of total shoots per culture. The same procedures used during the first subculture were repeated for four times (4 subcultures) to record the second, third and fourth subculture data (total shoots per culture and shoot formation and shoot length per explant).

The collected data of total shoots per culture, shoots formation and total shoot length per explant arranged in table with 12 explants for each combination of explants density and medium volume per culture and the data converted to table with three replicates by summation the data of each 4 explants and recorded as the first subculture data of total shoots number per culture. The total shoots per culture table converted to shoot formation

rate per explant by dividing each replicate by 4. Table for shoot length per explant was established in similar way. The shoot length of all shoots of each 4 of the 12 explants were summed and divided by total shoots obtained from those 4 explants to establish a table of three replicate for all of the combination of explants density and medium volume per culture. The tables obtained after each subculture for each parameter (shoot formation rate and shoot length per explant) combined in one table including all the combinations of explants density, medium volume and subcultures and subjected to three ways analysis of variance and the means significant tested by Duncan Multiple Range test at p 0.05 using SPSS statistical package No 11.0.

The total shoots per culture table converted to table of expected total after consecutive subcultures and used for statistical analysis and estimation of total cost and cost per shoot. The total shoots obtained at each replicate of the first subculture on the table of total shoot per culture were average to obtain the total shoot of the first subculture of each combination of explants density and medium volume per culture. The total shoot of the first subculture multiplied by the total shoots obtained at each replicate of the second subculture and averaged to estimate the total shoot expected to be obtained after two consecutive subcultures. The total of two consecutive subcultures multiplied by the total shoot obtained at each replicate of the third subculture and averaged to obtain the total expected after three consecutive subcultures. Total after 4 subcultures estimated in similar ways. The table of expected total subjected to three ways analysis of variance and the means significant tested by Duncan Multiple Range test at p 0.05 using SPSS statistical package No 11.0.

2.3. Rooting

2.3.1. Auxin types, concentrations and shoot ages

Full strength MS medium (1800 ml) was prepared from stock solutions, supplemented with sucrose at 30 g/l and divided into 9 beakers marked No 1 to 9. No hormone was added to beaker No. 1 and IBA at 0.5, 1.0, 1.5 and 2.0 mg/l were added to beakers No 2 to 5 and NAA at 0.5, 1.0, 1.5 and 2.0 mg/l added to beakers 6 to 9 respectively. The media pH was adjusted to 5.7 and the content of each beaker was dispensed into 9 glass jars (20 ml each), to each 0.14 gram of agar was added. The jars were closed with autoclavable lids and autoclaved for 25 minutes at 121^0 C and 1.5 kg/cm ^{2}. Smooth cayenne shoots were separated from 6-month old stock cultures and cultured individually one shoot per each jar. After 75 days of incubation, the shoots were removed from the cultures and the number of rooted shoots and the roots number per shoot were counted and the root length and plantlets height measured. Tables for number of rooted shoots, root number and root length per shoot and plantlets height for all treatments and 9 jars were constructed and converted to tables with three replicates using the average of each three jars. The root length per shoot was computed by summation of the root length per shoot and dividing by root number per shoot. The experiment was repeated after 4 months using shoots from 10 month old Smooth cayenne stock cultures. The tables of the same parameter from both experiments were combined in one table. The data subjected to three ways analysis of variance as function of hormone types, concentrations and shoot ages and the significant of means tested by Duncan Multiple Range Test at probability level of 0.05 using SPSS statistical package No. 11.

2.3.2. Auxins types, concentrations and cultivars

Four liters of MS medium was prepared from stock solutions, supplemented with sucrose at 30 g/l, divided into 11 beakers each received 360 ml of the medium and marked No. 1 to 11. No hormone was added to beaker No. 1 and IBA at 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l was added to beakers No.2 to 6. Similar concentrations of NAA added to beakers No 7 to 11 respectively. The pH was adjusted to 5.7 and the content of each beaker was dispensed into 18 glass jars (20 ml each) and marked with the same number of beakers plus capital letter S on 9 jars and M on the others. Agar (0.14 grams) was added to each jar. The jars were closed with autoclavable lids and the medium autoclaved at 121^0 C and pressure of 1.5 kg/ cm^2 for 25 minutes. Stock cultures of Smooth cayenne and Moris (6 months old) were used as source of explants. One shoot from Moris stock was cultured on each tube marked with M letter and one from Smooth cayenne stock on each tube marked with S letter. The cultures incubated under constant temperature of 25^0 C and 16 hours of light, After 75 days, cultures were removed from the incubation room for counting the number of rooted shoots and roots per shoot and measuring of root length and plantlets height. For each rooting parameter, the results of each three culture tubes of the same treatment were averaged and used as one of three replicate per treatment. Data subjected to analysis of variance and Duncan Multiple Range Test for treatments mean significance at probability level of 5 % using SPSS statistical package No. 11.

2.3.3. MS strengths and cultivars

One and half liters of full strength MS medium were prepared from stock solutions and divided into 720, 360 and 180 ml in 3 beakers marked with letter F, H and Q respectively. The medium in beaker H and Q completed to 720 ml using distilled water to obtain medium of half and quarter strength and sucrose at 30 g/l added to each of the three beakers. The content of each beaker divided into 4 glass jars (180 ml each) marked with

written on the beakers and numbered 1 to 4. No hormone added to jars No 1, IBA at 0.5 and 2.0 added to jars No 2 and 3 respectively and NAA at 1.0 mg/l added to jars No 4. The pH adjusted to 5.7 and 1.26 of agar added to each jar and the jars closed and the medium aurtoclaved at 121^0 C and pressure of 1.5 kg/ cm ^{2} for 25 minutes. The content of each jar divided under laminar into 18 culture tubes, marked with the same jar mark and letter S added to 9 and M to the other 9 tubes. One shoot from Moris stock cultures was cultured per each tube marked with M and one shoot from Smooth cayenne stock was cultured per tube marked with S letter. The cultures incubated under constant temperature of 25^0 C and 16 hours of light provided by cool white lamps. After 60 days, cultures were removed from the incubation room and shoot of each culture (jar) were picked out for counting the number of rooted shoots and roots per shoot and measuring root length and plantlets height. For each rooting parameter, the results of each three culture tubes of the same treatment were averaged and used as one of three replicate per treatment. Data subjected to analysis of variance and Duncan Multiple Range Test for treatments mean significance at probability level of 5 % using SPSS statistical package No. 11.

2.3.4. Shoot sizes and shoots density per culture

Full strength MS medium (600 ml) was prepared from MS powder in 1-liter glass bottle, enriched with sucrose at 30 g/l, IBA at 2.0 mg/l, the pH was adjusted to 5.7 and 4.2 grams of agar was added to the bottle and the medium was autoclaved for 25 minutes at 121^0 C and 1.5 kg /cm^2. The medium was dispensed under laminar into 60 culture tubes each received 10 ml of medium using sterilized syringe and each three culture tubes marked with No 1 to 20. Stock cultures of Moris pineapple were used as source of shoots. Multiple shoots complex were removed out of cultures under air flow laminar, placed on sterilized Petri dish, separated into individual shoots and arranged according to length into groups of 5, 10, 15 and 20 mm long shoots. The 5 mm long shoots were cultured at density

of 1, 2, 3, 4, and 5 shoots on the culture tubes No 1 to 5 and the 10, 15 and 20 mm long shoots were cultured at density of 1,2, 3, 4 and 5 shoots on the culture No 6 to 10, 11 to 15 and 16 to 20 respectively. Cultures were incubated under photoperiod of 16 hours of light and constant temperature 25 ^{0} C. After 60 days, the shoots were removed out of the cultures for counting the number of rooted shoots, root number per shoots and measuring roots length and plantlets height. The data were converted to average per shoot by dividing by the shoots density per culture. Each culture tube was used as a replicate and the data subjected to analysis of variance and mean separation using SPSS statistical package No. 11.

2.3.5. pH, sucrose concentrations and medium states

One and half liter of MS medium was prepared from MS powder, enriched with IBA at 0.5 mg/l and divided into 4 beakers each received 312 ml and marked A, B, C, and D. Sucrose at 10, 20, 30 and 40 g/l were added to beakers A, B, C and D respectively. The content of each beakers divided equally (78 ml) into 4 glass jars marked with the same beaker symbol plus 1, 2, 3 and 4. The pH of glasses number 1, 2, 3 and 4 adjusted to 5.0, 5.7, 6.0 and 6.5 respectively. Then the content of each glass was divided equally (26 ml) into 3 glasses marked with same glass symbol plus S, H and L and 0.18 and 0.09 grams of agar added to jars marked with S and H respectively and no agar was added to glass marked with L. The glasses were closed with autoclavable plastic lid and the medium was sterilized at 121^{O} C and 1.5 kg/ cm^{2} for 25 minutes. The content of each glass jar was dispensed under laminar into 3 culture tubes (8 ml each) marked with same glass mark. Three shoots from Moris stock cultures were cultured per each culture tubes. Cultures were incubated under 16 hours of light and constant temperature of 25 ^{0} C for 60 days. After 60 days, the cultures were removed from the incubation room, the shoots picked out of the

cultures and placed over squared paper for counting the number of rooted shoots and root number per shoot and measuring root length and plantlet height. Each tube was considered as one replicate and tables of three replicates for each rooting parameter were established by dividing by 3 (shoot density per culture). Analysis of variance and Duncan Multiple Range Test for significant of treatments at $p \leq 0.05$ were conducted using SPSS statistical package No. 11.

2.3.6. Sucrose concentrations and incubation periods

Half strength MS medium (450 ml) was prepared from MS powder, supplemented with IBA at 2.0 mg/l, divided into 6 glass jars (75 ml each) marked 1 to 6 and sucrose at 5, 10, 15, 20, 25 and 30 g/l were added to each glass jar respectively. The medium was adjusted to pH 5.0 and autoclaved at 121^0 C and 1.5 kg/ cm^2 for 20 minutes. The content of each glass jar was dispensed into 12 culture tubes (6 ml each) under laminar floor using sterilized syringe and the tubes marked with same glass jar mark. Shoots from Moris stock cultures were cultured at density of five shoots per culture. The cultures were incubated under constant temperature (25^0 C) and 16 hours of light provided by florescent lamps for different incubation periods (30, 45, 60 and 75 days). After 30 days of incubation, three culture tubes of each sucrose treatment were taken for data collection. The shoots were picked out to and the number of rooted shoots and number of roots per shoot were counted and the roots length and plantlet heigh were measured. Table for each rooting parameter of the different sucrose concentrations with three replicates was established and named the 30 days incubation tables. After 45, 60 and 75 days, three cultures tubes of each sucrose concentration were taken out of the culture room and table for each rooting parameter of the different sucrose concentrations at each incubation period was established as were done after 30 days incubation. The tables obtained for the same parameter after each incubation period were combined in one table containing all combinations of sucrose and incubation

periods and used for analysis of variance and testing the significant of means by Duncan Multiple Range Test at $p \leq 0.05$ using SPSS statistical packageNo.11.

2.4. Acclimatization

2.4.1. Substrate types and mixing ratio

After rooting in agar solidified full strength MS medium enriched with sucrose at 30 g/ and NAA at 1.0 mg/l for 75 days, *in vitro* rooted Smooth cayenne shoots (300 shoots) were washed, cleaned from agar and planted in pots (10 x 5 cm) filled with peat (1 : 0), sand (0 : 1) alone and 8 different mixing ratio of 5 and 1 part of peatmoss and 1, 2, 3 and 4 of sand sand (5: 1; 5: 2; 5: 3; 5: 4; 1 : 1; 1: 2; 1: 3; 1: 4). Each mixing ratio used to prepare 6 pots and 5 plantlets were planted in each pot. The pots were saturated with water, enclosed by polyethylene and placed under shadehouse conditions. After 15 days, the polyethylene enclosure was partially opened and after 21 days was removed and the plantlets were watered. After 75 days of the polyethylene removal, the plantlet heights were measured and the leaves were counted. The plantlets together with the substrates were removed out of the pots and placed in basin full of water to remove the substrate without damaging the root system. The plants were plot dried using filter paper and soft tissues, weighted, root counted and the length of roots were measured. The data of each parameter arranged in table of 6 replicates each replicate average of 5 plantlets and subjected to analysis of variance and Duncan Multiple Range test at $p \leq 0.05$ for testing the significant of means using SPSS statistical package No. 11.

2.4.2. Polyethylene cover and incubation periods

After rooting for 75 days in agar solidified full strength MS medium enriched with sucrose at 30 g/l and IBA at 0.5 mg/l, Moris pineapple plantlets (150 shoots) were transferred to pots filled with sands (15 plantlets per pot). Half of the plantlets (75

plantlets) were covered with polyethylene sheets and the other half were not covered and kept in room under normal room light and temperature (tropical region). The cover was partially opened after 10 days and completely removed after 21 days and the pot moved to shade house, natural day light and temperature of tropical area (32^0 C and 12 hour day light). The acclimatization experiment was repeated using shoots that were rooted in liquid half strength MS medium enriched with IBA at 2.0 and different concentrations of sucrose for different incubation periods. After rooting for 30, 45, 60 and 75 days, the plantlets (90 plantlets) were removed from the culture tubes and transferred (irrespective of the plantlets height, root number and length) to pots filled with sand (15 plantlets per pot), saturated with water, enclosed within polyethylene sheet and kept under room conditions. The polyethylene cover was removed after 15 days, the plantlets were transferred to shade house under natural day light and temperature (32^0 C, 12 hours day), watered every 7 to 10 days. After 60 days of acclimatization survival percentage were counted. The survival data were arranged in tables according to the different factors as covered and uncovered, liquid and solid media and the results were analyzed using t-test at p 0.05. The results of the second experiment were also arranged in tables of 4 incubations periods and 6 replicates, each replicate average of 15 plantlets, and subjected to analysis of variance and Duncan Multiple Range Test at $p \leq 0.05$ using SPSS statistical package No.11.

2.4.3. Rooting treatments and plantlets quality

Moris pineapple shoots (108 shoots) were rooted in solid and liquid MS enriched with different concentrations of IBA (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l). The cultures were numbered 1 to 108. Numbers starting from 1 to 9 were assigned to liquid medium without hormone, 10 to 18 to liquid medium enriched with IBA at 0.5 mg/l and continue up to 99 – 108 which assigned to solid medium enriched with IBA at 2.5 mg/l and incubated for 60 days under 16 hours of light and constant temperature of 25^0 C. The plantlets were

removed out of culture tubes, placed over square paper for counting rooted shoots, roots number per shoot and measuring roots length and plantlets height and placed individually in used culture tubes having the same serial number of the treatments (1-180) so each plantlet could be traced back to the type of the rooting treatment it received and the rooting results of that rooting treatment (root number, root length, plantlet height).. The plantlets were transferred to tray cells filled with garden soil mix and layout started from a marked cell on the tray and followed predetermine direction in accordance with a drown sketch. Hence, each plantlet on the tray could be traced back to type of rooting treatments and plantlet quality. The tray was enclosed within polyethylene sheets and placed under room temperature (tropical area) for 21 days. Each three plantlets were then transferred to pots (10 x 5 cm.) filled with compost and each pot was marked to facilitate tracing the plantlets to the rooting treatments and plantlets quality (the results of rooting treatment, root number, root length, plantlet height). The pots were placed under shade house and natural day light and temperature (12 hours long day and 32^{0} C). Plantlets were watered every 4 to 8 days and after 60 days the plantlets survival percentage was counted. The data were arranged in tables according to the different factors as rootless and rooted, hormone treated and hormone free, liquid and solid media. The plantlets from solid and from liquid media were arranged also into four categories of plantlet height, ($\leq$ 30 mm), (31- 40 mm), (41- 50 mm) and (51- 60 mm) and four categories of roots number, (rootless), (1- 2 roots), (3- 4 roots), (5- 6 roots) per plantlet. The data subjected to analysis of variance; t-test and Duncan Multiple Range Test using SPSS statistical package No. 11.

Results and Discussions

3.1. Sterilization and establishment of pineapple shoot tip

3.1.1 Effect of sterilant types and concentrations

Different treatments were tried for sterilization of terminal bud from Smooth cayenne fruit crown and Moris shoots and suckers. In almost all cases less than 30 % of the explants were successfully decontaminated and sprouted and the rest were lost either due to contamination or remained dormant. Terminal bud from suckers and shoots of Moris were more difficult to sterilize than terminal buds of crowns (Table, 3. 1). In all treatments higher percentage of clean cultures were obtained using crowns than suckers and shoots tip. Soaking the explants on Clorox (25 %) for 25 minutes was the best sterilization method for both cultivars and plant parts in which 50 % of the crown tip of Smooth cayenne and 22 % of suckers and shoot tips of Moris were clean and growing. On contrary, all explants treated with ethanol 50 % for 5 minutes followed by Clorox for 15 minutes were lost due to contamination. Using the other treatments, 11 to 22 % of shoots and suckers tips and 22 to 44 % of crown tips were successfully decontaminated and growing.

Clorox, $HgCl_2$ and ethanol are the most common sterilizing agents and were used each alone in single step sterilization and in sequence of two and three step sterilization procedures for sterilization terminal and lateral buds from suckers (Sripaoraya *et al*., 2003; Bhatia and Ashwath, 2002; Kofi and Adachi, 1993; Liu *et al*., 1989; Fernando, 1986), shoots (Teng, 1997) and slips of pineapple (Almeida *et al*., 2002; Wakasa, 1989). However, not only the percentage of success was rarely reported but also the reproducibility of these procedures is very low particularly when field grown plants were used as source of explants. Aydieh *et al.* (2000) reported 100 % clean growing explants using three steps sterilization, Clorox (30 %) for 20 minutes followed by $HgCl_2$ (0.1 %) for

30 seconds and ethanol (70 %) for 30 seconds. Singh and Manual (2000) reported 76 % clean lateral buds excised from fruit crown and underwent two step sterilization using Bavistin bleach (1 %) for 15 minutes and $HgCl_2$ (0.1 %) for 10 minutes while Zepeda and Sagawa (1981) reported 40 %. in two step sterilization using Clorox (10 %) before excision of lateral buds and Clorox (1 %) after excision of lateral buds from crown each for 60 minutes. Wakasa (1989) suggested Clorox (10 %) for 10 minute as the best compromise between injured explants using Clorox (20 %) for 20 minutes and contamination using Clorox (5 %) for 10 minutes. Similar sterilization procedures were used (Table, 3.1), but the percentage of clean and growing cultures was very low. Obtaining low percentage of clean and growing explants not only hindered investigation and optimization of establishment of primary explants but also prolong the time required to obtain stock culture large enough to start multiplication and commercial production.

3.1.2. Effect of BAP concentrations on establishment of pineapple shoot tip

In all reported pineapple tissue culture studies, the investigation of establishment of primary explants were always cut short probably because of contamination problem. Since obtaining of 100 % clean cultures is very difficult, the effect of factors hormone for instance, in the establishment of primary may accomplished by repeating of the establishment experiment until fixed number of clean cultures large enough for valid comparison is obtained. To compare the effect of BAP concentrations on establishment of crown tips of Smooth cayenne, explants were sterilized using Clorox (25 %) for 2 5 minutes and the experiment repeated several time till a sum of 9 cultures (clean and growing, clean but nongrowing and contaminated but growing cultures) was obtained. All of the crown tips that were not contaminated and some of those that were contaminated sprouted and proliferated in all BAP concentrations (Figure, 3. 1). However, the highest

shoot formation (7 shoots per explant) obtained in medium enriched with BAP at 2.25 mg/l and decreased to 2 shoots per explant as the BAP concentration increased to 5.25 mg/l

Mathew and Rangan (1979) reported that singly applied KN resulted in more shoot formation than combination of three hormones. However, they favored combination of three hormones because it resulted in higher establishment percentage of lateral bud culture than singly applied KN. Bhatia and Ashwath (2002) reported 100% establishment of Smooth cayenne laterals MS enriched with BAP at 1.0 mg/l.. However, in medium enriched with BAP at 0.025 and 2.25 mg/l, the establishment declined to 68 % and 20 % respectively. Hirimburegama and Wijesinghe (1992) noticed that the crown tip of Smooth cayenne could be established in MS enriched with NAA at 0.02 mg/l alone and in medium enriched with combination of BAP at 2.25 mg/l and IAA at 0.18 mg/l. Complete removal of BAP did not effect the establishment. However, when the concentration of BAP of the combination lowered to 0.25 mg/l, the explants failed to grow. At proper concentration, combination of two hormones**,** BAP and NAA (Rahman *et al*., 2001) and three hormones, KN, NAA, IBA (Khatun *et al*., 1997), all primary explants were established while at other concentrations the establishment declined to less than 15 %. Devi *et al.* (1997) reported that primary explants of pineapple could not be established on medium containing singly applied NAA or IAA at 0.1 mg/l and a combination of NAA at 0.1 and KN at 1.0 mg/l. However, all explants grew when the NAA concentration was increased to 1.0 mg/l.

Beside, the effect of hormone types and concentrations, successful establishment of bud culture was also affected by other factors as cultivar, medium states and intactness and physiological stage of the bud itself. Broomes and MacEvan (1994) reported that the percentages of sprouting buds after 30 days in liquid medium was 80 % while in solid medium was 63 %. None of Giant Kew sprouted in hormone free medium (Khatun *et al.,* 1997) and better establishment of Perola in MS enriched with BAP at 2.0 and IAA at 1.0

Table.(3.1). Effect of sterilization treatments and parts of mother plant on the percentage of clean primary explants

Sterilization treatments	Cultivars and (parts)	
	S. cayenne (Crown)	Moris (Suckers)
	% of clean explants	
Clorox (25 %) for 25 minutes	56	22
$HgCl_2$ (0.1 %) for 1 minute; Clorox (10 %) for 15 minutes	44	22
Clorox (25 %) for 25 minutes; $HgCl_2$ (0.1 %) for 1 minute	33	22
Clorox (10 %) for 15 minutes	22	11
Clorox (25 %) for 25 minutes; Clorox (10 %) for 15 minutes	33	11
Clorox (25 %) for 25 minutes; $HgCl_2$ (0.1 %) for 1 minute; Clorox (10 %) for 15 minutes	22	11
$HgCl_2$ (0.1 %) for 5 minutes	22	11
Ethanol (50 %) for 5 minutes; Clorox (10 %) for 15 minutes	0.0	0.0

Experiment was repeated several times. Each time 5 to 9 primary explants were used per each treatment.

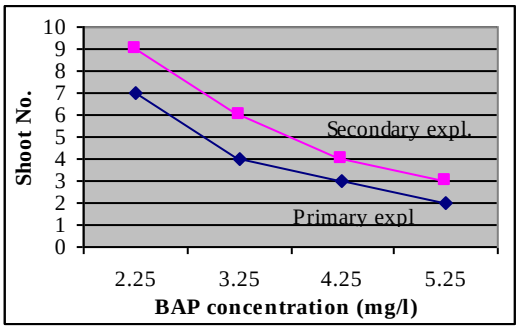


Figure (3.1) Effect of BAP concentrations on the proliferation of primary (crown tip) and secondary (in vitro shoots) explants of Smooth cayenne pineapple

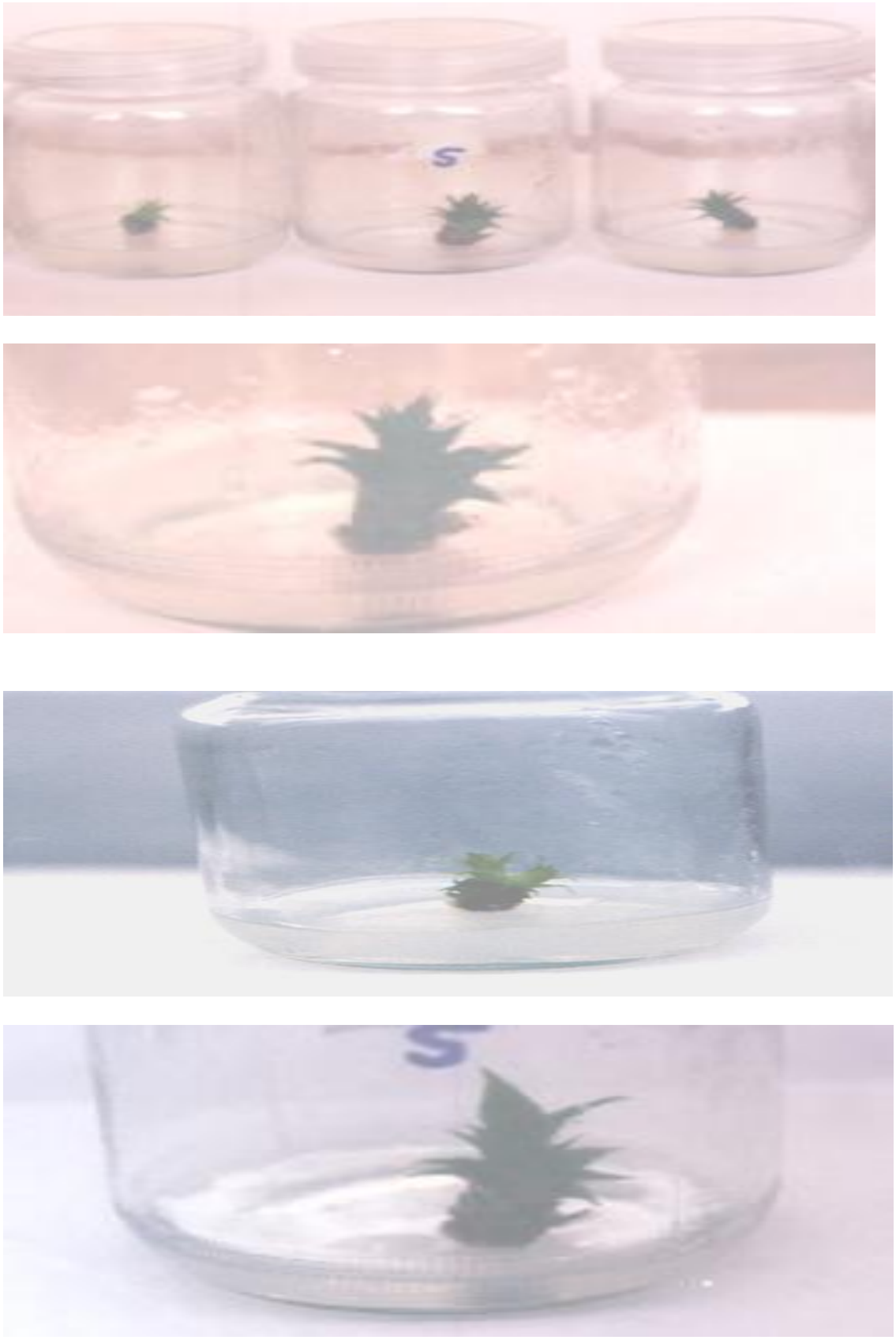


Plate (3.1). Established Smooth cayenne shoot tip in solid MS medium

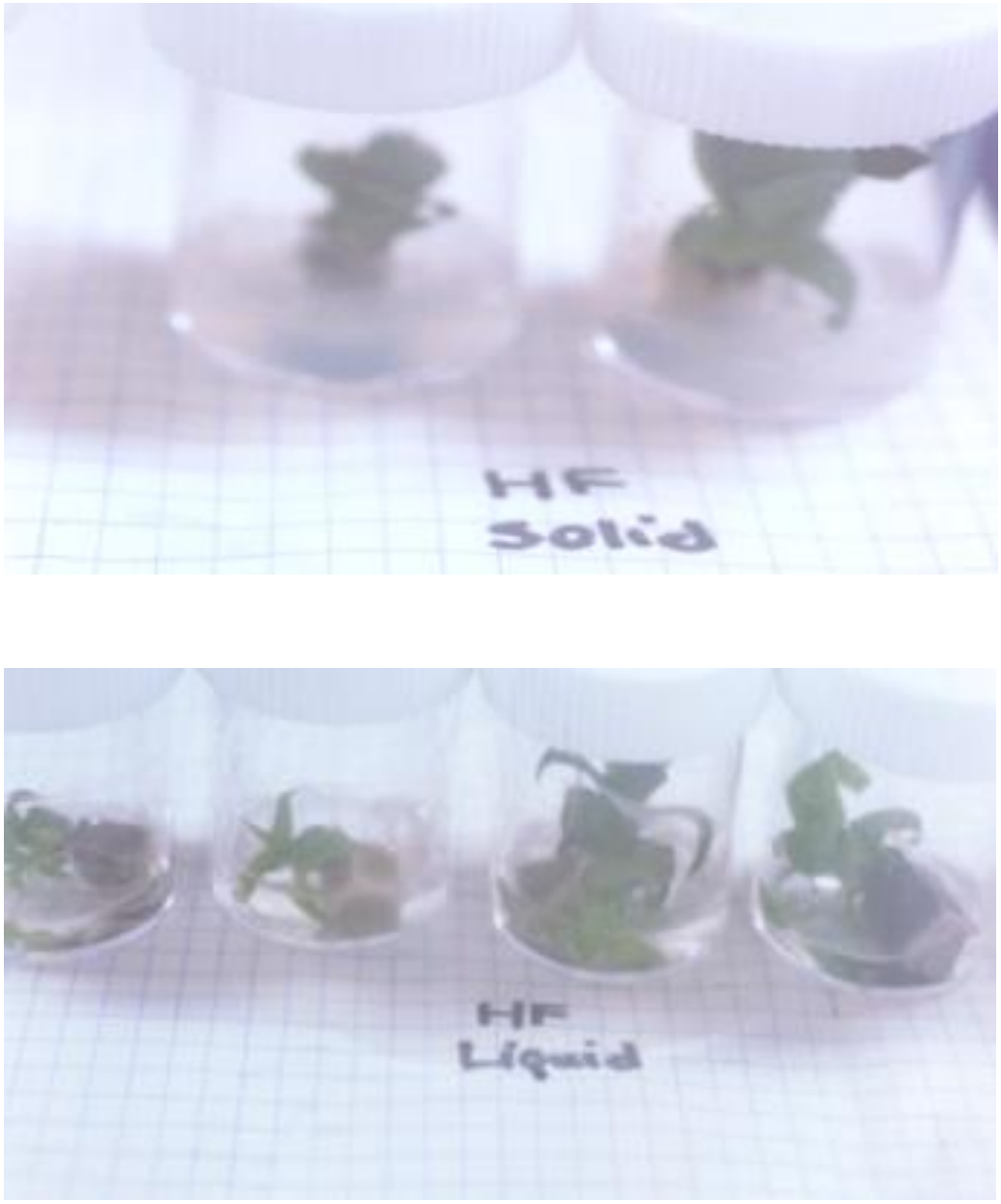


Plate (3.2). Established Moris shoot tips in solid and liquid MS medium (hormone free).

mg/l while Primavera better established in BAP at 3 and IAA at 1.0 mg/l (Almeida *et al.*, 1997). Firoozabady and Gutterson (2003) reported development of 2 to 3 shoots per primary explant within 30 days, while Mathew and Rangan (1979) reported formation of 6 to 8 shoots per explant in 8 weeks. Generally, a one month period is good enough for establishment of primary explants. However, for maximum shoot formation per primary and to secure large number of shoots to start the following multiplication stage, the establishment stage should be extended to two and three months. The results (Figure, 3.1) showed that the crown tip of Smooth cayenne could be established in solid MS enriched with BAP. However, the rate of shoot formation varied at different BAP concentrations.

3.2. *In vitro* multiplication of Pineapple

3.2.1. Effect of Benzylaminopurine and Indole acetic acid in establishment and multiplication of Smooth cayenne

Both primary (shoot-tips) and secondary explants (*in vitro* obtained shoots) of Smooth cayenne grew, proliferated and showed similar pattern of response to singly applied BAP. Highest shoot formation of both types of explants (7 shoots per primary and 9 shoots per secondary) obtained at 2.25 mg/l and decreased as the concentration increased to 5.25 mg/l (Figure, 3.1). At each concentration, the crown tips showed lower tendency for shoot formation than *in vitro* obtained shoots**.** ANOVA analysis (Table, 3.2) showed that shoot formation of the secondary explants were mainly affected by BAP ($p < 0.0002$) and interaction of BAP and IAA ($p \leq 0.0001$). The interaction of BAP and IAA was of cubic polynomial pattern ($p \leq 0.0001$) while the main effect of BAP followed quadratic pattern ($p \leq 0.045$). The effect of IAA on the shoot formation was not significant ($p \leq 0.487$). Overall BAP concentrations, the shoot formation at different concentrations of IAA were equal (6 shoots/explants). On contrary, overall IAA concentrations, there were different

shoot formations rate at different BAP concentrations. BAP at 2.25 mg/l resulted in the highest average (8 shoots). Increasing the BAP to 3.25, 4.25 and 5.25 mg/l reduced the shoot formation by 2 and 3 respectively. The highest shoot formation per explant (9 shoots) obtained in response to single application of BAP at 2.25 mg/l, combination of BAP at 2.25 plus IAA at 0.75 mg/l and IAA at 1.75 mg/l and combination of BAP at 3.25 plus IAA at 1.75 mg/l (Table, 3.3). Other concentrations of BAP whether applied alone or in combination with IAA did not result in shoot formation higher than that obtained when BAP at 2.25 mg/l was singly applied.

Whether incubation of IAA with BAP increased or decreased the shoot formation depended on the concentration of both hormones (Table, 3. 3). Compared to single application of BAP, inclusion of IAA at 0.75 mg/l with BAP at 3.25 mg/l decreased the number of shoot by 2 shoots (6 to 4 shoots), but in combination with BAP at 4.25 mg/l increased the shoot formation by 4 shoots (4 to 8 shoots). However, the same IAA concentration did not affect the rate of shoot formation when used in combination with BAP at 2.25 mg/l (9 shoots on both) and at 5.25 mg/l (3 shoots on both). Similar, compared to singly applied BAP at 3.25 mg/l, inclusion of IAA at 1.75 mg/l resulted in formation of three more shoots. Shoot formation on medium containing IAA at 2.75 plus BAP at 2.25 mg/l were 4 shoots less than that on medium containing BAP alone. On the contrary, using the same concentration of IAA resulted in 3 shoots more when applied in combination with BAP at 5.25 mg/l. Inclusion of IAA at 3.75 mg/l with BAP at 2.25 mg/l and with BAP at 3.25 mg/l decreased the shoots by 2 and 3 respectively compared to single application of BAP. On the contrary, the same concentration of IAA (3.75 mg/l) increased the shoot formation by 4 and 5 when applied together with BAP at higher concentration of BAP (4.25 and 5.25 mg/l).

Single application of BAP at 2.25 mg/l in agar solidified MS medium induced more shoots (9 shoots) than that reported using combination of three hormone (Mathew and Rangan, 1979; Mathew *et al.*, 1976), two hormones (Firoozabady and Gutterson, 2003; Boxus *et al.*,1991; Fitchet, 1993) and single application of BAP at 1.0 (Aydieh *et al.*, 2000), at 1.5 (Almedia *et al*., 2002) and 2 mg/l (Bhatia and Ashwath, 2002). However, these reported shoot formation rate is very low compared to the 25 shoots obtained using combination of BAP and NAA (Rahman *et al*., 2001). Several researchers agreed that the two (Firoozabady and Gutterson, 2003; Wakasa, 1989) and three hormones combinations (Sripaoraya *et al.*, 2003) were required only during the establishment stage. During multiplication, they recommended single application of BAP.

Mathew and Rangan (1979) reported that the proliferation was mainly controlled by cytokinin and the auxin only enhanced the BAP effect. Table 3.2 showed that IAA had no direct significant effect on shoot formation but through interaction with BAP. However, Table 3.3 showed that the auxin not only enhanced but also suppressed the cytokinin effect depending on the concentrations of both hormones. Compared to singly applied BAP at 2.25 mg/l none of IAA concentrations improved the proliferation. Inclusion of IAA at 2.75 mg/l with BAP at 2.25 mg/l reduced the shoot formation by 4 shoots compared to 9 shoots obtained when BAP at 2.25 mg/l applied alone (> 44 % loss of the proliferation capacity). IAA at 3.75 and at 1.75 caused 22 and 11 % loss on the effectiveness of BAP while IAA at 0.75 had no effect. On the contrary, all of the IAA levels improved the effectiveness of BAP if the BAP was applied at higher concentration of 5.25 mg/l and that improvement increased as the IAA concentration increased. IAA at 1.75, 2.75 and 3.75 mg/l increased the shoot formation by 25, 100 and 166 % respectively. The effectiveness of BAP at 3.25 and 4.25 mg/l, on the other hand, could either be improved or suppressed depending on the concentration of IAA (Table, 3.3). Inclusion of IAA at 0.75 and 3.8 mg/l with BAP at 3.25

mg/l reduced respectively the rate of shoot formation compared to BAP alone from 6 to 4 (30 % loss of effectiveness) and from 6 to 3 (50 %) shoots while IAA at 1.75 mg/l improved the rate from 6 to 9 shoots (50 % enhancement of effectiveness) and IAA at 2.75 mg/l resulted in same rate as that of BAP alone. On contrary, when used in combination with BAP at 4.25 mg/l, IAA at 0.75 mg/l increased the rate from 4 to 8 (100 % enhancement) and IAA at 3.75 mg/l increased the rate from 4 to 6 shoots (50 % enhancement) while IAA at 1.75 and 2.75 mg/l reduced the shoot formation from 4 to 3 shoots (25 % loss of effectiveness). The highest shoot formation of all treatments obtained when BAP at 2.25 mg/l was used alone. Compared to BAP at 2.25 mg/l, increasing of BAP concentration up to 5.25 mg/l and using of other combinations of IAA and BAP resulted in loss of proliferation capacity. Hence, the importance of IAA inclusion seemed to be overcoming declining of proliferation caused by single application of BAP at high concentrations (3.25, 4.25 and 5.25 mg/l). In other word, if the right concentration of BAP was used, there would be no need for inclusion of IAA in the medium. Singly applied BAP at 2.25 mg/l could be used for both of establishment and proliferation of Smooth cayenne crown tip. IAA concentrations which interacted negatively and those which interacted positively could be selected for further study to monitor the metabolic changes within the explants and elucidation of the mode action of the hormones.

The different in shoot formation and the IAA suppression and enhancement of BAP effect indicated two different interaction mechanisms between IAA and BAP. Regression analysis at constant concentration of one hormone and different concentration of the other showed different R^2 values (Table, 3.3). About 99% of variation on shoot formation on medium devoid of IAA was caused by the differences in BAP concentrations. However, when IAA at 0.75 mg/l was included, the differences in BAP concentration accounted only for 4 % ($R^2 = 0.04$) of the variation observed in the shoot formation. This

implies that the overall interaction of IAA at 0.75 mg/l with BAP concentrations neutralized the BAP effect on the process of shoot formation. Following the same approach, IAA at 1.75, 2.75 and 3.75 mg/l caused 38%, 82% and 23% loss on the BAP effect on the shoot proliferation process respectively. These variations on the percentage of BAP accountability for shoot formation reflect how strongly the interaction between IAA and BAP was. IAA at 0.75 mg/l had the highest interaction capability with BAP. Similar, comparing the R^2 values at constant BAP and different concentrations of IAA indicated that BAP at 4.25 mg/l had the highest interaction (98%) and BAP at 5.25 mg/l the lowest interaction capability (1%) with IAA. Correlation coefficient indicated a significant negative relation between the concentrations of singly applied BAP and shoot formation of both primary and secondary explants (r = - 0.96). Regression coefficient (R^2 = 0. 91) indicated that 90 % of the differences in shoots formation was caused by different in BAP concentrations. The functional relation between the concentration and shoot formation was linear and the high value of correlation (r) and regression coefficient (R^2) indicated that the shoot formatiom per explant over BAP range 2.25 to 5.25 mg/l could be expected using the following models.

Shoot No. (Primary explant) = 10.08 – 1.6 (BAP conc.) R^2 91 p 0.0438

Shoot No. (Secondary explant) = 13.10 – 2.0 (BAP conc.) R^2 95 p 0.0240

Function relation between concentration of hormone (Deora and Shekhawat, 1995), length of shoot after one week of incubation (Salehi and Khosh-Khui, 1996) and the shoot formation capability was suggested for estimation number of shoots of *Capparis decidua* and miniature roses culture after one month of incubation respectively.

Table (3.2). Trend analysis of the main and interaction effect of BAP and IAA on the *in vitro* shoot formation of Smooth cayenne pineapple

Source	df	Pr > F
BAP	3	0.0002 **
Linear	1	0.0001 **
Quadratic	1	0.0458 *
Cubic	1	0.8442
IAA	4	0.4875
BAP*IAA	12	0.0001**
Linear	1	0.0001 **
Quadratic	1	0.0381*
Cubic	1	0.0001 **
Error	38	
Total	59	

Table (3.3) Effect of BAP and IAA combinations on the *in vitro* shoot formation of Smooth cayenne pineapple

	IAA (mg/L)						
BAP (mg/L)	0	0.75	1.75	2.75	3.75	Average	R^2
				Shoots / explants			
2.25	9a	9a	8 ab	5 bcdef	7 abcd	8A	0.6244
3.25	6 abcde	4 def	9a	6 abcde	3 f	6 B	0.4663
4.25	4 def	8ab	3 f	3 f	6 abcde	5 B	0.0784
5.25	3 f	3 f	4 def	6 abcde	8ab	5 B	0.9938
Average	6 NS	6 NS	6 NS	5 NS	6 NS		
R^2	0.995	0.0377	0.62	0.167	0.77		

The results were means of 9 shoots individually cultured in 20 ml of agar solidified (7g/l) full strength MS medium containing 30 g/l sucrose and adjusted to pH 5.7 after 60 days of incubation.
Means followed by the same letters were not significantly different as tested by Duncan's Multiple RangeTest at $p \leq 0.05$.
NS (No significant)

3.2.2. Multiplication and growth pattern of pineapple Smooth cayenne over 105 days of incubation on different hormone treatments

At each incubation period there were different optimal hormone treatments for shoot formation and total weight and length of shoots (Table, 3.4). After 15, 30 and 45 days of incubation, the best treatment for shoot formation was combination of BAP at 3.25 and IAA at 1.75 mg/l and the least effective treatment was combination of BAP at 3.25, IAA at 3.75 mg/l. They resulted in the highest formation of 4, 11 and 12 shoots and the lowest formation of 1, 3 and 7 shoots per explant respectively. After 60 and 90 days, single application of BAP at 2.25 mg/l was the best treatment resulting in 15 and 25 shoots per explant respectively while after 75 and 105 the best treatments were combinations of BAP at 2.25, IAA at 0.75 mg/l and BAP at 2.25, IAA at 1.75 mg/l resulting in 24 and 31 shoots per explant respectively. The least effective treatment after 60, 75, 90 and 105 was combination of BAP at 3.25, IAA at 3.75 mg/l resulting in 8, 9, 10 and 10 shoots per explant respectively. There was no increase in shoot formation after 75 days of incubation in medium enriched with BAP at 3.25, IAA at 3.75 mg/l and after 90 days of incubation in medium enriched with BAP singly applied at 2.25 mg/l and combination of BAP at 2.25 with IAA at 0.18 mg/l. However, the shoot formation in medium enriched with BAP at 2.25, IAA at 1.75 mg/l, BAP at 3.25, IAA at 1.75 mg/l and medium enriched with BAP at 2.25, IAA at 1.75, GA_3 at 1.0 mg/l increased as the incubation period increased up to 105 days. In fact, over 30 % of the shoot formation (11 of 31 shoots) in response to BAP at 2.25, IAA at 1.75 mg/l occurred after 90 days of incubation. According to maximum shoot formation, the incubation of the medium enriched with BAP at 2.25, IAA at 1.75 mg/l should be extended to 105 days while for medium enriched with BAP at 2.25 mg/l alone and in combination with IAA at 0.175 and IAA at 0.8 mg/l, the incubation should be terminated after 90 days.

Except in case of the medium enriched with combination of BAP at 3.25, IAA at 3.75 mg/l, where no increase in weight occurred after 90 days of incubation, the total weight of shoots was generally increased as the incubation increased to 105 days (Table, 3.4). However, the optimal and the least effective treatment varied at different incubation periods. The highest total weight after 15, 30, 45, 90 and 105 days of incubation were 0.44, 0.99, 1.45, 4.69 and 5.91 grams, all obtained respectively in medium enriched with combination of BAP at 3.25, IAA 1.75 mg/l while after 60 and 75 days, the heaviest weight were 2.13 and 3.48 grams obtained in medium enriched with combination of BAP at 2.25, IAA at 1.75, GA_3 at 1.0 mg/l and medium enriched with combination of BAP at 2.25, IAA 0.175 mg/l respectively. The lowest total shoots weight after shorter incubation periods of 15, 30 and 45 days, were 0.20, 0.63 and 0.96 grams all obtained respectively in medium enriched with combination of BAP at 2.25, IAA 1.75, GA_3 at 1.0 mg/l while at longer incubation period of 60, 75, 90 and 105 days, the lowest weight were 0.95, 1.18, 2.09 and 2.17 grams all obtained respectively in medium enriched with combination of BAP at 3.25, IAA at 3.75 mg/l. On the other hand, the highest total length of shoots after 15 (28 mm), 30 (55 mm) and 45 (86 mm) days of incubation obtained in medium enriched with BAP 3.25, IAA 1.75 mg/l while the highest total length after 75 (244 mm) and 90 (272 mm) days of incubation obtained in medium enriched with BAP at 2.25, IAA at 0.75 mg/l. The highest total length of shoots after 60 days (135 mm) obtained in medium enriched with BAP alone at 2.25 mg/l while after 105 days, medium enriched with combination of BAP at 2.25, IAA at 1.75 mg/l resulted in the highest total shoot length (406 mm). At shorter incubation periods of 15, 30 and 45 days, the best treatment for all parameters (shoot formation, total length and weight) was combination of BAP at 3.25, IAA at 1.75 mg/l while at longer incubation of 60, 75 and 90 days, the best treatment was

combination of BAP at 2.25, IAA at 0.75 mg/l. After 105 days of incubation the best was BAP at 2.25, IAA at 1.75 mg. L^{-1} (Table, 3 4).

The different treatments showed different shoot formation patterns (Table, 3 5). For each treatment, there were alternative pulses of high and low shoot formation and gain in total length and weight of shoots. However, the period at which the high and low pulses occurred and the magnitude of the pulses varied among treatments. The highest shoot formation per two weeks in medium enriched with BAP alone at 2.25 mg/l, combination of BAP at 2.25, IAA at 0.75 mg/l and BAP at 2.25, IAA at1.75 mg/l, GA_3 at 1.0 mg/l was 8, 10 and 7 shoots respectively all occurred in the same period between the 60- 75 days of the 105 days long incubation while the highest shoot formation in medium enriched with combination of BAP at 2.25, IAA at 1.75 mg/l and BAP at 3.25, IAA 1.75 mg/l was 11 and 7 shoots occurred in the period between the 90- 105 and the 15- 30 days of the 105 days incubation respectively. On the other hand, the lowest shoot formation in medium enriched with BAP alone at 2.25 mg/l; combination of BAP at 2.25, IAA at 0.175 mg/l and BAP 2.25, IAA at 0.75 mg/l was 2, 1 and 1 shoot occurred at the period between 75- 90, 60- 75 and 75 – 90 days of incubation respectively. In all of these treatments no shoot formation occurred in the last 15 days of incubation (90- 105 days). For the medium enriched with combination of BAP at 2.25, IAA at 1.75 mg/l, the lowest shoot formation per two weeks was 1 shoot occurred in the periods between 31- 45 and 76- 90 days. Furthermore, while in case of medium enriched with combination of BAP at 2.25, IAA at 1.75 mg/l and medium enriched with BAP at 3.25, IAA at 1.75 mg/l, there were 3 pulses of high shoots formation (5, 7 and 11 shoots) and (7, 3 and 3 shoots) occurred at the period between 15- 30, 60- 75 and 90- 105 days of incubation respectively, shoots in the medium enriched with BAP at 3.25, IAA at 3.75 mg/l showed only one period of high shoot formation (4 shoots) occurred between 30 and 45 days of incubation after which the shoot formation was only

one shoot per each two weeks. The other treatments showed two pulse of high shoot formation occurred in the period between 15- 30 and 60- 75 days of incubation (Table, 3 5).

The increase in total shoot length and weight at each two week period showed also pattern of high and low pulses. The highest gain in total length per two weeks in medium enriched with BAP at 2.25, IAA at 1.75 mg/l, medium enriched with BAP at 2.25, IAA at 1.75, GA_3 at 1.0 mg/l and medium enriched with BAP alone at 2.25 mg/l was 207, 96 and 76 mm respectively all occurred in the period between 90 and 105 days of incubation. For medium enriched with BAP at 2.25, IAA at 0.75 mg/l and BAP at 3.25, IAA at 1.75 mg/l, the highest gain in total shoots length was 118 and 59 mm occurred at the period between 60 to 75 and 75 to 90 days of incubation respectively. The highest gain in total weight per two weeks, on the other hands, was 3.11, 2.43 and 1.81 grams occurred respectively in the period between 90 -105, 75 - 90 and 60 - 75 days of incubation in medium enriched with BAP at 2.25, IAA at 1.75 mg/l, medium enriched with BAP at 3.25, IAA at 1.75 mg/l and medium enriched with BAP at 2.25, IAA at 0.175 mg/l. In medium enriched with BAP alone at 2.25 mg/l and medium enriched with BAP at 3.25, IAA at 3.75 mg/l, there were only one peak of total shoots weight gain (1.20 and 0.91 grams) occurred at the last 15 days (90 to 105) and between the 75 and 90 days of incubation respectively. Medium enriched with BAP at 2.25, IAA at 0.175 mg/l and BAP at 2.25, IAA at 0.75 mg/l resulted also in one peak each (1.81 and 1.52 grams) but in both media the peak occurred in the same period between the 60 and 75 days of incubation. On the contrary, the shoots cultured in medium enriched with BAP at 2.25, IAA at 1.75 mg/l, BAP at 3.25, IAA at 1.75 mg/l and with BAP at 2.25, IAA at 1.75, GA_3 at 1.0 mg/l showed two peaks of gain in the total shoots weight. In all of these treatments, one peak (3.11, 1.22 and 1.30 grams) occurred at the last 15 days of the 105 days of incubation. However, the second peak (0.91, 2.43 and

1.17 grams) occurred at different periods of incubation (60 to 75, 75 to 90 and 45 to 60 days respectively).

In almost all of the reported multiplication studies of pineapple different hormone treatments were compared at one fixed incubation period ranged from 15 to 84 days. Using of one fixed incubation period neither serves propagators nor physiologist goals. In spite of its outmost important for assessment, management, cost of production, number of possible multiplication cycles per year and consequently the final total of shoot production, feasibility studies and physiological investigation, in all reported studies selection of incubation was an arbitrary decision. Little attention was paid to comparison of the effect of different incubation periods and selection of proper ones. As this study demonstrated, the optimal hormone at one incubation period could even be better if the incubation was prolonged for extra two or more weeks and vice versa and the use of fixed incubation period could come short from discovering the real best treatment. Table 3.4 showed that the shoot proliferation capacity could last for a minimum of 75 days of continuous exposure to singly applied BAP and in combination with IAA. However, if the incubation extended to 90 days, explants treated with BAP at 2.25 mg/l plus IAA at 0.75 mg/l lost its shoot formation ability while those treated with other hormone combinations remained capable of shoot formation. At prolonged incubation of 105 days, only three (BAP at 2.25, IAA at 1.75 mg/l with and without GA_3 at 1.0 mg/l and BAP at 3.25 mg/l plus IAA at 1.75 mg/l) of the seven treatments remained effective. In fact 30% of the total shoots (11of 31 shoots) in response to combination of BAP at 2.25 mg/l plus IAA at 1.75 mg/l were produced during the last15 days of the 105 days of incubation. In addition, irrespective of how long the explants remained responsive, the total shoots at the end of any of the incubation periods and the shoot formation at every two weeks interval varied at different hormone treatments. The total shoots after 105 days of incubation ranged from a lowest of

11 to a highest of 31 shoots (Table, 3. 4) and the shoots formation at each two weeks intervals ranged from none to 11 shoots (Table, 3. 5).

It is important to mention that even if the different treatments resulted in equal total shoots, it had different shoot formation pattern. Explants treated with BAP alone at 2.25 mg/l and combination BAP at 2.5 and IAA at 0.75 mg/l both resulted in 25 shoots after 105 days of incubation. However, the biweekly shoot formations were 1, 6, 3, 4, 8, 2, 0 and 1, 5, 2, 5, 10, 1, 0 shoots respectively. Similar, explants treated with BAP at 3.25, IAA at 1.75 mg/l and those treated with BAP at 2.25, IAA at 1.75 mg/l plus GA_3 at 1.0 mg/l produced 20 and 19 shoots after 105 days of incubation but had different shoot formation pattern. It is interesting that while in case of treatment BAP 3.25, IAA 3.75 mg/l, it took 105 days to produce 10 shoots, the same amount of shoots produced on only just 15 days in case of treatment BAP at 2.25 plus IAA at 1.75 and IAA at 0.75 mg/l. It is clear that the shoot formation occurred at alternative pulses of high and low and different treatments differed on the time and magnitudes of those pulses. In other word, the different hormone treatments differ not only on the shoot formation per explant but also in the time when the treatments exerted its highest effect and when it regained or lost its effectiveness. Kofi and Adachi (1993) reported different optimal hormone treatments at different incubation of 30 and 60 days. In medium enriched with NAA at 0.5 mg/l, inclusion of either BAP or KN induced the highest while 2iP induced the lowest shoot formation of Sugar loaf pineapple incubated for 30 days. However, if incubated for 60 days, BAP resulted in highest shoot formation, followed by 2iP while KN became the least effective hormone. The length of multiplication cycle had also impact on the best hormone type and concentrations for *in vitro* rooting (Danso *et al*., 2008). Escalona *et al.* (1999) reported a relation between explants density and incubation period and total of shoots and frequency of shoot sizes and

found that the shoot formation per week increased up to the 7th week and declined afterward.

Longer incubation period resulted in more shoots than shorter incubation (Table, 3. 4) and consequently would decrease the cost of jars and medium but increase the electricity cost of incubation. The incubation should be extended as long as the increases in shoot formations lead to decline in the cost per single shoot. If the treatment terminated earlier or later than it should be, the cost per shoot would be higher than an expected selling price. The majority of the researchers suggested an incubation period of 30 to 45 days. However, a tentative estimation of the cost after one cycle of multiplication (Table, 3. 6) showed that none of the treatments resulted in shoot cost less than 5 cents unless the incubation was at least 75 days. After 75, 90 and 105 days of incubation, the cost of production in case of singly applied BAP at 2.25 mg/l and combination of BAP at 2.25 and IAA at 0.75 mg/l was 5 cents. However, the cost per shoot in case of treatment BAP at 2.25, IAA at 1.75 mg/l could be as low as 4 cents but only if the incubation was 105 days long. In both treatments, the cost per shoot after the most commonly suggested incubation period of 30 and 45 days was two times higher (10 and 11 cents per shoot). Hormones differ in their effectiveness as *in vitro* shoot formation inducer and different incubation periods would result in different electricity cost (photoperiod and air condition). Hence, for reduction of cost, the best combination of hormone and incubation period hormone treatment is compromise between obtaining the highest effect of the hormone (shoot formation rate) and lowest electricity bill.

The different in total weight at each two weeks and at the end of incubation (Table, 3. 4 and 3.5) indicated a possibility of successful *ex vitro* acclimatization if the shoots were directly transferred at the period in which the highest gains of weight were occurred. The weight of shoots was not used before as parameter of assessment of different hormone

treatments or incubation periods of pineapple. However, using *Spathipuyllum floribundum*, Ramirez *et al*. (2001) reported that hormonal treatments that resulted in heavier shoots during multiplication stage resulted also in higher survival during acclimatization. The main purpose of pineapple tissue culture so far is propagules production. However, due to expected increase in the application of the pineapple bromelain for medical and industrial uses, the weight may soon become an important parameter for selection of best treatment. BAP at 2.25 and at 3.25 mg/l in combination with IAA at 1.75 mg/l and longer incubation of 105 days seemed to be a best choice for biomass production (6 gm/explant).

The goal of external addition of hormone and other compounds is to change the internal hormonal balance of the explants in favor of shoot formation. However, unless the internal balance is identified, tissue culture would remain a mere try and error experiment and different treatments could be claimed as the best for shoot formation. Chemical and histological analysis revealed that exogenous application of BAP and NAA to pineapple leaf base (Hamasaki *et al*., 2005; Mercier *et al*., 2003) and etiolated node (Souza *et al*., 2003) caused a sharp increase in the endogenous content of IAA and Isopentenyladenine (Ip) within a week and that coincide with shoot formation. Shoots could be seen by naked eye within two weeks. The pulses of high and low shoot formation observed in this study are reflection of an endogenous changes in hormonal content, enzymes activity and mineral content of the explants and medium that need to be identified. Histological, chemical and radioactive techniques are usually used in investigation of metabolism and physiological events underline differentiation and developmental process. These techniques are very complicated, expensive and required highly skillful personnel for conducting and interpretation of the results. Most importantly, deriving of valuable information from these techniques depend mainly in selecting of proper time of application. Treatments of contradicting high and low shoot formation pulses and the

Table (3.4) Effect of hormone treatments and incubation periods on the *in vitro* shoot formation rate, length, weight and total shoots of Smooth cayenne pineapple

Hormones treat. (mg/l)	**Incubation periods (Days)**						
	15	30	45	60	75	90	105
Shoots per explants							
BAP(2.25)	2 rq	8 lmno	11 ijkl	15 fghi	23 bcd	25 b	25 b
BAP(2.25) IAA(0.175)	2 rq	6 lmnopr	10 jklm	13 ghik	14 ghij	17 efg	17 efg
BAP(2.25) IAA(0.75)	2 rq	7 lmnop	9 klmn	14 ghij	24 bc	25 b	25 b
BAP(2.25) IAA(1.75)	2 rq	7 lmnop	8 lmno	12 hijkl	19 def	20 cde	31 a
BAP(3.25) IAA(1.75)	4 oprq	11 ijkl	12 hijkl	13 ghik	16 efgh	17 efg	20 cde
BAP(3.25) IAA(3.75)	2 rq	5 noprq	8 lmno	8 lmno	9 klmn	10 jklm	11 ijkl
BAP(2.25) IAA(1.75) GA_3 (1.0)	1 q	3 prq	7 lmnop	9 klmn	16 efgh	17 efg	19 def
Total shoot length (mm)							
BAP (2.25)	18 uv	41 stuv	79 oprqstu	135 ijklmno	208 efgh	225 cdef	301 b
BAP (2.25) IAA (0.175)	18 uv	37 stuv	70 prqstuv	117 klmnopr	98 mnoprqs	168 fghijk	137 ijklmno
BAP (2.25) IAA (0.75)	18 uv	42 stuv	74 prqstuv	126 jklmnop	244 cde	272 bc	300 b
BAP (2.25) IAA (1.75)	18 uv	42 stuv	48 qstuv	108 lmnoprq	188 efghi	180 fghij	406 a
BAP (3.25) IAA (1.75)	28 tuv	55 qstuv	86 noprqst	104 lmnoprq	145 ijklmn	204 efgh	214 defg
BAP (3.25) IAA (3.75)	18 uv	16 v	40 stuv	56 qstuv	61 rqstuv	72 prqstuv	75 prqstuv
BAP(2.25) IAA(1.75) GA_3 (1.0)	15 v	18 uv	62 rqstuv	95 mnoprqs	160 ghijkl	153 hijklm	266 bcd
Total shoot weight (g)							
BAP (2.25)	0.28 kl	0.8 ijkl	1.36 fghijkl	1.65 efghijkl	2.25 defghij	3.03 bcdefg	4.23 bc
BAP (2.25) IAA (0.175)	0.27 kl	0.97 ijkl	1.1 hijkl	1.67 efghijkl	3.48 bcde	33.52 bcde	3.85 bcd
BAP (2.25) IAA (0.75)	0.28 kl	0.73 ijkl	1.45 fghijkl	1.66 efghijkl	3.18 bcdef	3.71 bcd	4.5 abc
BAP (2.25) IAA (1.75)	0.27 kl	0.93 ijkl	0.96 ijkl	1.75 efghijkl	2.66 cdefghi	2.99 bcdefgh	6.1 a
BAP (3.25) IAA (1.75)	0.44 jkl	0.99 ijkl	1.45 fghijkl	1.80 efghijkl	2.26 defghij	4.69 ab	5.91 a
BAP (3.25) IAA (3.75)	0.27 kl	0.51 jkl	0.89 ijkl	0.95 ijkl	1.18 ghijkl	2.09 defghijkl	2.17 defghijk
BAP(2.25) IAA(1.75) GA_3 (1.0)	0.20 k	0.63 jkl	0.96 ijkl	2.13 defghijkl	2.18 defghijk	2.18 defghijk	3.48 bcde

Data represent means of 9 shoots individually cultured on 20 ml of agar solidified (7 g/l) full strength MS medium enriched with sucrose at 30 g/l). Means of the same parameter followed by the same letter were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range Test.

Table (3.5). Effect of the hormone treatments on the biweekly increase in shoots formation, length, weight and total shoot of Smooth cayenne pineapple over 105 days of incubation

Hormone treatments (mg/l)	**Growth per two weeks over 105 days of incubations**						
	(1-15)	(16-30)	(31-45)	(46–60)	(61–75)	(76-90)	(91-105)
Shoots per explant.							
BAP(2.25)	1 f	6 bcde	3 def	4 cdef	8 abc	2 ef	0 f
BAP(2.25) IAA(0.175)	1 f	4 cdef	4 cdef	3 def	1 f	3 def	0 f
BAP(2.25) IAA(0.75)	1 f	5 cdef	2 ef	5 cdef	10 ab	1 f	0 f
BAP(2.25) IAA(1.75)	1 f	5 cdef	1 f	4 cdef	7 abcd	1 f	11a
BAP(3.25) IAA(1.75)	4 def	7 abcd	1 f	1 f	3 def	1 f	3 def
BAP(3.25) IAA(3.75)	1 f	3 def	3 def	0 f	1 f	1 f	1 f
BAP(2.25) IAA(1.75) GA_3(1.0)	1 f	2 ef	4 cdef	2 ef	7 abcd	1 f	2 ef
Total shoots length (mm)							
BAP(2.25)	3 f	23 def	38 cdef	56 bcdef	73 bcde	17 def	76 bcd
BAP(2.25) IAA(0.175)	3 f	19 def	33 cdef	21 def	35 cdef	8 def	37 cdef
BAP(2.25) IAA(0.75)	3 f	24 def	32 cdef	52 cdef	118 b	28 def	28 def
BAP(2.25) IAA(1.75)	3 f	24 def	6 ef	60 bcdef	63 bcdef	28 def	207 a
BAP(3.25) IAA(1.75)	13 def	27 def	31 cdef	18 def	41 cdef	59 bcdef	10 def
BAP(3.25) IAA(3.75)	3 f	4 f	19 def	15 def	5 ef	11 def	4 f
BAP(2.25) IAA(1.75) GA_3(1.0)	0 f	3 f	44 cdef	33 cdef	65 bcdef	10 def	96 bc
Total shoots weight (g)							
BAP (2.25)	0.08 b	0.52 ab	0.56 ab	0.29 b	0.60 ab	0.78 ab	1.20 ab
BAP (2.25) IAA (0.175)	0.07 b	0.7 ab	0.13 b	0.57 ab	1.81 ab	0.04 b	0.33 b
BAP (2.25) IAA (0.75)	0.08 b	0.48 ab	0.69 ab	0.21 b	1.52 ab	0.53 ab	0.79 ab
BAP (2.25) IAA (1.75)	0.07 b	0.66 ab	0.03 b	0.79 ab	0.91 ab	0.33 b	3.11 a
BAP (3.25) IAA (1.75)	0.24 b	0.55 ab	0.46 ab	0.36 b	0.46 ab	2.43 ab	1.22 ab
BAP (3.25) IAA (3.75)	0.07 b	0.24 b	0.38 b	0.06 b	0.23 b	0.91 ab	0.07 b
BAP (2.25) IAA (1.75) GA_3(1.0)	0.00 b	0.43 b	0.33 b	1.17 ab	0.06 b	0.00 b	1.30 ab

Data represent means of 9 shoots individually cultured on 20 ml of agar solidified (7 g/l) full strength MS medium enriched with sucrose at 30 g/l.

Increase on shoots formation per explant, total length and weight of shoots at each increment of two weeks were calculated by substraction of the values obtained at one incubation from that of the previous one. (Incubation for 30 – Incubation for15 days) and (Incubation for 45 – incubation for 30 days) and so on.

Means of the same parameter followed by the same letter were not significantly different at $p \leq 0.05$ according to DMRTest.

Table (3.6). Estimated variable cost per shoot of Smooth cayenne pineapple after different incubation periods in different hormone treatments

Hormone treatments (mg/l)	**Incubation periods** (Days)						
	15	30	45	60	75	90	105
	Cost/ shoot (RM)						
BAP (2.25)	0.35	0.10	0.08	0.07	0.05	0.05	0.05
BAP (2.25) IAA (0.175)	0.35	0.13	0.09	0.08	0.08	0.07	0.08
BAP (2.25) IAA (0.75)	0.35	0.11	0.10	0.07	0.05	0.05	0.05
BAP (2.25) IAA (1.75)	0.35	0.11	0.11	0.08	0.06	0.06	0.04
BAP (3.25) IAA (1.75)	0.17	0.07	0.07	0.08	0.07	0.07	0.06
BAP (3.25) IAA (3.75)	0.35	0.13	0.11	0.12	0.12	0.12	0.13
BAP (2.25) IAA (1.75) GA_3 (1.0)	0.69	0.26	0.13	0.11	0.07	0.07	0.07

RM Ringgit Malaysia 1 USA $ = RM 3.25.
The calculated cost included only the variable items of cost (Jars, MS, agar, sucrose, hormones, electricity cost of autoclave, laminar operation and incubation room and the wage of labors).

periods at which the contradicting responses occurred (Table, 3. 5) might help in determining the best times for applying of these investigation techniques.

3.2.3. Incubation periods and hormone treatments

The rate of shoot formation was increasing as the incubation period increased irrespective of the hormone treatment by about 3 shoots per explant per each two weeks for 75 days (Table, 3.7). The highest shoot formation after 30, 45, 60 and 75 days of incubation was 7, 10, 14 and 19 shoots respectively. However, at different incubation periods there were different optimal hormone treatments for pineapple proliferation. After 30 days of incubation, the best hormone treatments were BAP (2.25) IAA (0.18); BAP (2.25) IAA (0.75) and BAP (3.25) IAA (1.75). Each resulted in formation of 6 to 7 shoots per explant. When the incubation period was increased to 45 days, the best treatments were BAP singly applied at 2.25 mg/l and combination of BAP (3.25) IAA (1.75). Both resulted in formation of 10 shoots. After 60 and 75 days of incubation, explants cultured in media containing BAP (3.25) IAA (0.75) and BAP (3.25) IAA (1.75) produced the highest shoot formation per explant (14 and 19 shoots respectively). Each of the media containing BAP (3.25) IAA (1.75) and BAP (2.25) IAA (0.75) were one of the best at three incubation periods (excluding incubation for 60 and 45 days respectively). BAP singly applied at 2.25 mg/l was one of the best at two incubation periods (45 and 75 days) while BAP (2.25) IAA (0.18) and BAP (2.25) IAA (1.75) was one of the best at only one incubation period, 30 and 75 days respectively (Table, 3.7).

A rate of 4 (Zepeda and Sagawa, 1981) and 20 shoots (Bordoloi and Sarma, 1993) were obtained in 30 days of incubation. Similar, Bhatia and Ashwath (2002) and Sripaoraya *et al*. (2003) reported formation of 6 and 10 shoots after 45 days incubation. Be and Debergh (2006) and Almeida *et al*. (1997) obtained 9 shoots after 60 days and Teixeira *et al*. (2006) reported formation of 13 shoots after 75 days of incubation. However, none of

them investigated the effect of different incubation periods and hormone treatments on the the shoot formation per explant and total shoot production over subcultures. The shoot formation per explant (Table, 3.7) and the total shoot produced over four multiplication cycles (Table, 3.8) showed that different hormone treatments could result in equal shoot formation rate but different total shoot production. Using either one of the parameters for treatments assessments lead to same conclusion as long as the incubation period did not exceed 60 days. According to both parameters, singly applied BAP at 2.25 mg/l and combined application of BAP at 3.25, IAA at1.75 mg/l were the best hormone treatments at 30 and 45 days of incubation. However, at 75 days incubation, both treatments resulted in statistically equal shoot formation per explant (17 and 19 shoots) while the different in total shoots production was 53125 shoots. It is a very big difference that could not be overlooked and indicating that using of shoot formation rate is not proper parameter of assessment particularly if the goal is to develop system for mass production of propagules. Different optimal hormone treatment at different incubation was reported for hybrid tea rose (Arnold *et al*., 1995) and equal shoot formation rate but different total shoot were also observed in Paeony culture (Harris and Mantell, 1991).

Longer incubation period resulted in formation of more shoot per explant, higher total shoots and lower cost per shoots but required higher total cost (budget) than shorter incubation (Table, 3.8). Overall hormone treatments, a total of 722, 3698, 17751 and 57924 shoots at total cost of RM 324, RM 1129, RM 3670 and RM 8903 and cost per shoot of RM 0.35, RM 0.23, RM 0.19 and RM 0.15 could be produced after 4 consecutive subcultures of 30, 45, 60 and 75 days of incubation respectively. However, at different incubation period of 30, 45, 60 and 75 days, there were different optimal hormone treatments for pineapple proliferation. In other word, the choice of best combination of incubation period and hormone treatment is a matter of management of the cost and

targeted total shoot production. The highest total shoot production from explants incubated for 30 days was 1680 shoots obtained in medium enriched with combination of BAP at 2.25, IAA at 0.18 mg/l at total cost of RM 612 and cost per shoot of RM 0.24 while the minimum total shoots production after 75 days of incubation obtained in medium enriched with BAP at2.25, IAA at1.75, GA_3 at1.0 mg/l was ten times (10080 shoots) of the maximum total shoots obtained after 30 days of incubation but at higher total cost of RM 4398 and cost per shoot of RM 0.31. On the other hand, equal cost per shoot but different total shoots and total cost could be obtained at equal as well at different incubation periods using different hormone treatments. Equal cost of RM 0.15 per shoot was obtained after incubation for 45 days on medium enriched with BAP alone at 2.25 mg/l and in combination with IAA at 1.75 mg/l but at different total shoots (8470 and 3430 shoots) and total cost (RM 1842.37 and 749.54 respectively). Similar, equal cost per shoot (RM 0.12) was obtained after incubation for 60 days in MS medium enriched with BAP at 2.25, IAA at 0.75 mg/l and after incubation for 75 days in MS enriched with BAP alone at 2.25 mg/l and in combination with IAA at 0.175 and at 0.75 mg/l, but the total shoots was 32256, 68000, 24570 and 78750 shoots obtained at total cost of RM 5683.4, RM 11473.6, RM 4034.7 and RM 13359.5 respectively. The highest total shoots (121125 shoots), highest shoot formation per explant (19 shoots) and the lowest cost per shoot (RM 0.08) obtained from explants incubated for 75 days in medium enriched with BAP at 3.25, IAA at1.75 mg/l. However, obtaining that total would require investment of RM 13437.4 over half of it would be paid for electricity. The major cause of cost per shoot (Figure 3. 2) was electricity of incubation room (3.47 cents) followed by wages of labor for culturing (1.16 sents) and MS medium (1.11 cents). Measuring the shoot formation rate, and computing the expected total shoot production at different incubation period is very crucial for better assessment of treatments and management of mass production. It provides the propagator

with several alternatives selection of which would be decided according to target total and time of propagules delivery and available budget and facilities.

Mathew and Rangan (1979) said that incorporation of IAA merely enhanced the BAP effect on pineapple *in vitro* shoot formation. However, Hirimburegama and Wijesinghe (1992) noticed that IAA at low concentration enhanced the BAP effect but at high level completely blocked the BAP effect. The results (Table, 3.7) showed that the inclusion of IAA with BAP promoted the formation at certain concentrations and suppressed the proliferation at others depending on how long the incubation period was. Compared to singly applied BAP, addition of IAA at 0.175, 0.75 and 1.75 mg/l suppressed the shoot formation at 45 days of incubation. However, when the incubation shorten to 30 or extended to 60 days, IAA at 0.175 mg/l increased while at 75 day decreased the shoot formations and IAA at 0.75 mg/l had no effect. Compared to single application of BAP at 2.25 mg/l, incorporation of IAA at 1.75 mg/l together with BAP, suppressed the BAP promotion of shoot formation if the incubation was 45 days. However, if the incubation was shorted to 30 or extended to 60 and 75 days, incorporation of IAA neither suppressed nor promoted the BAP effect. Incorporation of IAA at 0.75 mg/l, on the other hand, enhanced the BAP effect if the incubation period was 30 and 60 days (1 shoot higher), suppressed the effect if the incubation was 45 (2 shoot less) and had no effect if the incubation extended to 75 days (equal rate). IAA at 0.175 mg/l increased the shoot formation obtained after 30 days of incubation by 2 more shoots but reduced the shoot formation obtained after 45 and 75 days of incubation by 2 and 5 shoots respectively. Similarly, compared to BAP at 3.25 plus IAA at1.75 mg/l, increasing the IAA concentration to 3.75 mg/l lowered the BAP promotion of shoot formation at 30, 45, 60 and 75 days of incubation by 1, 3, 4 and 8 shoots respectively. Incorporation of GA_3

Table (3.7). Effect of hormone treatments and incubation periods on *in vitro* multiplication (A), shoot length (B) and total shoots (C) over 4 subcultures of Smooth cayenne

Hormone treatments (mg/l)	**Incubation period** (Days)				
	30	**45**	**60**	**75**	Average
A. Shoots per explant					
BAP (2.25)	5 fg	10 cd	13 b	17 a	11 AB
BAP (2.25) IAA (0.175)	6 ef	8 de	13 b	12 bc	10 B
BAP (2.25) IAA (0.75)	6 ef	7 def	14 b	18 a	11 AB
BAP (2.25) IAA (1.75)	5 fg	8 de	13 b	18 a	11 AB
BAP (3.25) IAA (1.75)	6 ef	10 cd	12 bc	19 a	12A
BAP (3.25) IAA (3.75)	5 fg	7 def	8 de	11 bc	7 C
BAP (2.25) IAA (1.75) GA_3 (1.0)	3 g	6 ef	7 def	12 bc	7 C
Average	5 D	8 C	11 B	15 A	
B. Shoot length (mm)					
BAP (2.25)	6 cde	7 bcde	9 bc	9 bc	8 B
BAP (2.25) IAA (0.175)	6 cde	7 bcde	8 bcd	8 bcd	7 C
BAP (2.25) IAA (0.75)	7 bcde	7 bcde	9 bc	9 bc	8 B
BAP (2.25) IAA (1.75)	6 cde	5 de	8 bcd	10 b	8 B
BAP (3.25) IAA (1.75)	6 cde	8 bcd	8 bcd	9 bc	8 B
BAP (3.25) IAA (3.75)	5 de	6 cde	8 bcd	7 bcde	6 D
BAP (2.25) IAA (1.75) GA_3 (1.0)	8 bcd	8 bcd	11a	11a	10A
Average	7 C	7 C	8 B	10A	
C. Total shoots					
BAP (2.25)	384 e	8470 cde	25200 cd	68000 b	25513 A
BAP (2.25) IAA (0.175)	1680 de	2430 de	21294 cd	24570 cd	12493 B
BAP (2.25) IAA (0.75)	1134 de	2268 de	32256 c	78750 b	28602 A
BAP (2.25) IAA (1.75)	384 e	3430 de	27000 cd	92160 b	30743 A
BAP (3.25) IAA (1.75)	1056 de	6240 cde	13824 cde	121125 a	35561 A
BAP (3.25) IAA (3.75)	360 e	2100 de	2520 de	10780 cde	3940 B
BAP (2.25) IAA (1.75) GA_3 (1.0)	54 e	882 de	2160 de	10080 cde	3294 B
Average	722 C	3688 C	17750 B	57923 A	

Data were means of 9 shoots cultured individually in 20 ml of full strength agar solidified (7 g/l) MS medium enriched with sucrose at 30 g/l and adjusted to pH 5.7.
Means followed by same letters were not significantly different at $p \leq 0.05$ as screened by Duncan Multiple Range Test

Table (3.8). Total cost and cost per shoot of Smooth cayenne after 4 subcultures on different hormone treatments for different incubation periods

Hormone treatments (mg/l)	**Incubation period** (Days)			
	30	45	60	**75**
		Total cost (RM)		
BAP (2.25)	163.63	1842.9	5709.1	11473.59
BAP (2.25) IAA (0.175)	611.8	1246	4345.5	4034.739
BAP (2.25) IAA (0.75)	562.84	583.44	5683.4	13359.51
BAP (2.25) IAA (1.75)	289.57	749.75	4296.8	12986.41
BAP (3.25) IAA (1.75)	418.51	2350.7	3661	13437.36
BAP (3.25) IAA (3.75)	191.74	662.04	1065.5	2632.004
BAP (2.25) IAA (1.75) GA_3 (1.0)	32.895	466.39	929.93	4398.174
Average	324	1128.7	3670.2	8903.1
		Cost per shoot (RM)		
BAP (2.25)	0.30	0.15	0.16	0.12
BAP (2.25) IAA (0.175)	0.24	0.35	0.14	0.12
BAP (2.25) IAA (0.75)	0.33	0.17	0.12	0.12
BAP (2.25) IAA (1.75)	0.50	0.15	0.11	0.10
BAP (3.25) IAA (1.75)	0.27	0.25	0.18	0.08
BAP (3.25) IAA (3.75)	0.37	0.22	0.30	0.17
BAP (2.25) IAA (1.75) GA_3 (1.0)	0.44	0.36	0.31	0.31
Average	0.35	0.23	0.19	0.15

Smooth cayenne shoots were cultured at density of 1 shoot per glass jar containing 20 ml of full strength agar solidified (7 g/l) MS medium enriched with sucrose at 30 g/l and adjusted to pH 5.7..Medium were dispensed at 20 ml per glass jar and autoclaved at 121 ^{0} Cand 1.5 kg/l for 25 minutes and the cultures were kept under constant temperature 25 ^{0} C and 16 hour of light provided by cool white florescence lamps.
RM. Ringgit Malaysia (1 USA $ = RM 3.25).
The calculated cost included only the variable items of cost (Jars, MS, Sucrose, Agar, labor wages and electricity cost for operating autoclave, laminar and incubation room).

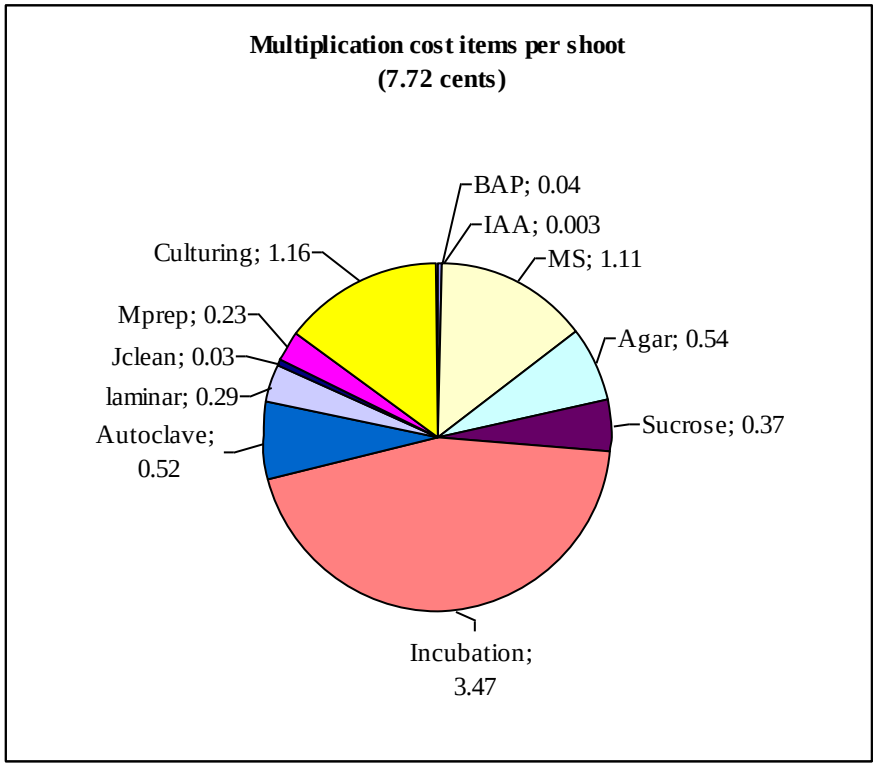


Figure (3.2) Partitioning of the multiplication cost items per shoot of Smooth cayenne after 4 consecutive subcultures. Shoots were cultured at density of one shoot on 20 ml of full strength agar solidified (7 g/l) MS enriched with sucrose at 30 g/l and incubated for 75 days under constant temperature 25 ^{0} C and 16 hour of light provided by cool white florescence lamps. Medium was dispensed at 20 ml per glass jar and autoclaved at 121 ^{0} C and 1.5 kg/ cm^{2} for 25 minutes
Mprep (medium preparation), Jclean (jar cleaning)
Culturing (separation and culturing of shoots).
Laminar, autoclave and incubation (electricity cost for operation of laminar, autoclave and incubation room)

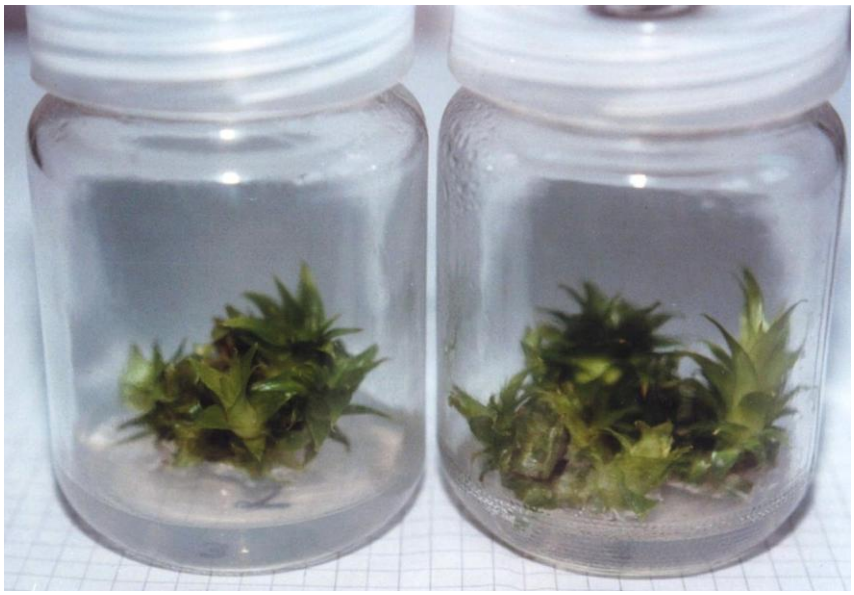


Plate (3.3) Multiplication of Smooth cayenne pineapple in agar solidified (7 g/l) full strength MS enriched with BAP at 2.25 mg/l (left) and combination of BAP at 3.25 and IAA at 1.75 mg/l (right)

together with BAP at 2.25, IAA at1.75 mg/l reduced the shoot formation at 30, 45, 60 and 75 days by 2, 2, 6 and 6 shoots respectively.

Obtaining of longer shoot is better than shorter shoots. Longer shoots are easy to handle, halved and is expected to have higher shooting and rooting capacity than shorter shoots. GA_3 was included to improve the shoot length. Inclusion of GA_3 and longer incubation resulted in longer shoots than shorter incubation. However, of all treatments the difference between tallest and shortest shoots was less than 5 mm (Table, 3.7) and that improvement in shoot length was at the expense of shoot formation per explant. Using of selected shoot size (20 to 25 ml) for multiplication, an average shoot length of 30 (Aydieh *et al*., 2000), 60 (Soneji *et al*., 2002b) and even 90 mm (Khan *et al.,* 2004) were reported during multiplication. The too short shoot obtained in this study could be attributed to using of different shoot size (6 to 25 mm long) rather than selective shoot size and reporting of average of 4 subcultures and to different cultivars and hormone treatments.

3.2.4. Incubation periods and subcultures

ANOVA analysis showed that the shoot formation per explant was significantly affected by subcultures ($p < 0.0001$), incubation periods ($p < 0.0001$) and interaction of subcultures and incubations ($p < 0.0199$). The total shoots over consecutive subcultures were also significantly affected by subcultures, incubation periods and interaction of both ($p < 0.0001$). However, while subcultures induced no direct significant effect ($p < 0.8106$) on shoot length, the shoot length was affected by incubation periods ($p < 0.0001$) and interaction of incubation periods with subcultures ($p < 0.0019$). Table 3.9 showed that the shoot formation average overall incubation periods for the first three subcultures (14, 13 and 12 shoots) were not significantly different. However, the fourth subculture caused 50% loss (7 shoots) on the shoot formation capability. Average overall subcultures, on the other hand, showed that short incubation for 30 days resulted in fewest and shortest shoots (6

shoots 6 mm long) and a longer incubation for 75 days resulted in the highest and tallest shoots (19 shoots 9 mm long). The shoots formation and shoot length after an intermediate incubation of 45 and 60 days long were not significantly different (10 and 12 shoots of 8 mm long), but it was higher than that of the 30 and lower than the 75 days of incubation (Table, 3.9).

Excluding the fourth subculture, a comparison of the shoot formation and shoot length over the first three subcultures showed that the nature as well as the magnitude of subcultures effect varied depending on the length of the incubation period and the number of subcultures. The shoot formation of the explants incubated for 30 days decreased over the first three subcultures from 11 to 6 and 4 shoots, respectively. However, this effect reversed when the explants were maintained on the medium for 75 days. Instead of decreasing, the shoot formation increased from 17 to 19 and 25 shoots. At 45 days of incubation, the negative effect of subculture appeared at the second to disappear by the third subculture while at 60 days the shoot formation remained the same (12 shoots) for the first two subcultures but increased to 16 shoots by the third subculture. At each subculture, a longer incubation produced more shoots than a shorter incubation; however, the difference becomes wider over repeated culturing (subcultures). The difference in shoot formation between explants incubated for 30 and those incubated for 75 days during the first subculture were 6, increased to 13 during the second and reaching a maximum of 21 at the third subculture before declining to 11 at the fourth subculture. Similar, the difference in shoots obtained after 60 and 30 days of incubation was 1 shoot at the first, increased to 6 shoots and 12 shoots at second and third and declined to 2 shoots by the fourth subculture. On the contrary, the difference between shoots formed at 60 and 75 days of incubation was continuously increased from 5 shoots at the first subculture to 7, 9 and 9 at the second, third and fourth subcultures. Similar, longer incubation resulted in longer shoots than

shorter incubation. Overall subcultures, the shoot length increased from 6 mm at 30 days of incubation to 8, 8 and 9 mm at 45, 60 and 75 days incubations. Compared to 30 days incubation, extending the incubation to 75 days improved the length at first, second and third subculture by 4, 3 and 4 mm, respectively. Average of shoot length overall incubations, on the other hand, remained almost unchanged with about 8 mm long at each subculture. Individual comparison showed that the subcultures improved the shoot elongation if the incubation was for a short period of 30 and 45 days and suppressed the increase in shoot length at a longer incubation of 60 and 75 days. By the fourth subculture, the shoots from explants incubated for 30 and 45 days were longer and those incubated for 60 and 75 days were shorter than the shoots formed during the previous subcultures (Table, 3.9).

Converting the average of shoot formation at different incubation periods (Table, 3.9) to percentage of shoot formation at each 15 days increment (Figure. 3.3) and rate per week showed that shoot formation over 75 days incubation occur in alternative cycles of high and low pulses, the sequence and magnitude of which was affected by subcultures. In the first subculture, the majority of shoots (65%) formed during the first 30 days (11 of 17 shoots) with a rate of 2.6 shoots per week. Then the shoot formation process almost ceased for the next 30 days (6%) at a rate of 0.5 per week before it resumed during the last 15 days (60–75 days) of incubation where formation of 24% of the shoots (4 of 17 shoots) at a rate of 2 shoots per week occurred. On the contrary, formation of the shoot during the fourth subculture delayed for 60 days where 60% of shoots (9 of 15 shoots) at a rate of 4.5 shoots per week appeared during the last 15 days of the 75 days long incubation, about 27% of the shoots (4 of 15 shoots) at a rate of 0.9 shoot per week appeared in the first 30 days then the process almost ceased (7%) for 30 days with a rate of 0.6 shoot per week. During the third subculture, there were two peaks of shoot formation one started after the

first 30 days whereby 32% of shoots (8 of 25 shoots) formed in the period between 30 and 45 days at a rate of 4.0 shoots per week and the second after 60 days where 36% of shoots (9 of 25 shoots) formed in period between 60 and 75 days at a rate of 4.5 shoots per week. During the second subculture about one third (32 and 37%) of the shoot formation occurred at the first 30 days and the period between 60 and 75 days of incubation but at different shoot formation rate of 1.4 and 3.5 shoots per week, respectively.

Table 3.10 showed that at each subculture, incubation for 30 days resulted in the lowest and incubation for 75 days resulted in the highest total of shoots. By the fourth subculture, the shorter incubation period of 30 days resulted in the lowest total (1056 shoots) and the longer incubation of 75 days resulted in the highest total (121125 shoots) while 45 and 60 days incubation resulted in an intermediate totals. Total of shoots obtained after 4 subcultures from the explants incubated for 60 days was two times (13824 shoots) of that (6240 shoots) incubated for 45 days. However, the total of each of the first three subcultures were not significantly different. Comparing total of shoots in terms of months showed that different total of shoots but equal time and equal total of shoots but at different time could be obtained and vice versa. After 4 months, a total of 1056 shoots could be obtained using a 30 days long incubation and 4 subcultures while the 60 days incubation and 2 subcultures produced only 144 shoots. In a 6 months period, the incubation for 45 days (4 subcultures) trippled the total of shoots (6240 shoots) compared to total obtained (2304 shoots) using a 60 days incubation (3 subcultures). On the other hand, the total shoots obtained after 6 months (6240 shoots) using 45 days incubation and four subcultures was statistically equal to a total obtained after 7.5 months (8075 shoots) using 75 days incubation and three subcultures (Table, 3.10). Similar, the total shoots obtained after 3 months (264 shoots) using 30 days incubation and three subcultures were not significantly different from that obtained after 5 months (323 shoots) using 75 days

incubation and two subcultures. After three subcultures of each, the total obtained after 6 months (2304 shoots) using 60 days incubation and 4.5 months (1460 shoots) using 45 days incubation were not significantly different. Obvious, in case of equal total shoot production, shorter time would be naturally favored over longer time.

Longer incubation resulted in higher shoot formation per explant (Table, 3.9) and total shoot production at lower cost per shoot than shorter incubation, but at higher expenditure (Table, 3.10). After 30 days of incubation, the total cost at the third and fourth subculture were RM 82.34 and RM 320.81 while after 75 days incubation the total cost were RM 514.2 and RM 12894.5 respectively. However, the cost per shoot after 30 days was RM 0.25 and RM 0.27 while after 75 days the cost per shoot was RM 0.05 and RM 0.08. Limiting the subcultures to three subcultures resulted in lowest cost per shoot (RM 0.05) but the total shoots (8075 shoots) was 15 times less than that (121125 shoots) obtained after four subcultures at cost of RM 0.08 per shoot. Table 3.11 showed that the major cause of cost of *in vitro* multiplication was electricity of incubations (RM 4208.5) followed by cost of jars (RM 3876.0), labor wages for culturing of shoots (RM 1402.66) and MS medium (RM 1346.56). From each one Ringgit spent during multiplication 31.32 cents was for paying the electricity bill of incubation room, 28.85 cents for buying glass jars, 10.44 cents to labor for separation and culturing of shoots and 10.02 cents to buy the MS medium. Since the glass jars are reusable item, it was not included in estimation of cost per shoot (Figure, 3.2) but it should be included in total cost and estimation of budget.

Both physiologist and propagator are interested in tissue culture; however, they have different goals and concerns about different parameters. While the former use the time as tool to monitor occurrence of events, the second sees the time as factor for product management. The length of incubation and number of multiplications (subcultures) before the shoot formation sharply decline is very crucial for the propagator, the rate (average per

explant per unit of time) and shoot formation pattern is essential for the physiologist. If the results of an interesting study were reported as average per explant or per unit of time, the propagator would convert it to a total per each and over consecutive cycles to facilitate his calculations and the scheduling of certain tasks. Similar, the results reported as average per explant would be converted by the physiologist to rate and pattern over time to contemplate about mechanism and the proper time at which histological and biochemical investigations should take place. Investigating the changes in the shoot formation average and the total over different incubations and repeated cycles of multiplications would serve the interest of both.

In the few cases in which different incubation periods were compared, pineapple shoots incubated for 60 days incubation not only resulted in more shoot formation but also require different optimal cytokinin type and concentration (Kofi and Adachi, 1993) and explant density (Escalona *et al*., 1999) from that incubated for 30 days. Using hardy and hybrid tea rose, Arnold *et al*. (1992) also reported different hormone treatments optimal for different incubation periods. Harris and Mantell (1991) reported significant differences in rate and the total shoots of each subculture of Paoeny shoot tip incubated for 3, 4 and 5 weeks and subcultured for four times. Since the rate and optimal treatment varied at different incubations, using a fixed incubation would limit the propagator to a specific incubation that may not suit his plan. Similar, as the rate varied over the subcultures, expectation of a total of consecutive subcultures based on an overall average rate would either come short or would overestimate the possibly obtainable total shoots. The risk of high cost per unit of production due to either over investing or unexpected shortages of shelving space, vessels and labor is extremely high.

Reporting the obtained total shoots of consecutive subcultures over different incubation periods will provide the propagator with room for maximizing the total production and

minimizing cost and time and for selection of incubation period that meet the delivery due time. Table 3.10 indicates that a different total of shoots could be produced at an equal period of time and an equal total at different period times depending on the incubation and subcultures combinations. Hence, several alternatives are available for the propagator to choose from according to cost of subculture, incubation and time of delivery. A better plan could be tailored to fit small or big companies according to their available budget, facilities, and market demand. A decision of whether to start with 1, 10 or 100 explants, adopting of 4 (30 days long incubation), 2 (45 days incubation), 1.5 (60 days incubation) or 1.2 (75 days incubation) production cycles per year could be made to accomplish certain total production target, within certain time in accordance with available budget. To take the advantages of low cost per shoot (RM 0.05), starting with 15 shoots and limiting the subcultures to 3 subcultures would result in total equal to that obtained after 4 subcultures and 75 days incubation (121125 shoots) at lower total cost (RM 7713) and starting with 25 shoots would result in higher total shoots (201875 shoots) at equal total cost (RM 12894.53). Cost is always seen as a main problem, but to reduce the cost the first step is to identify the major factor of the cost. Figure 3.2 indicated that substantial reduction in multiplication cost could be achieved by investigating the effect of photoperiod and temperature on multiplication. Complete elimination of incubation electricity bill could be achieved by testing outdoor multiplication.

The physiologist uses the rate (average per explant per unit of time) to monitor the pattern of response and select proper treatment and incubation period. To maintain a high shoot formation rate the explants should transfer to fresh medium, just before the time the rate start to decline. Escalona *et al*. (1999) reported that the rate of shoot formation of Smooth cayenne pineapple per week increased over incubation before it declined by the seventh week. Converting the shoot formation average per incubation (Table, 3.9) to a rate

per week showed that the increase in rate over the incubation period occurred only if the explants were previously subcultured for more than one time. Over 75 days incubation, the shoot formation rate of explants at the first and the fourth subculture was on contrary decreasing from 2.56 and 0.9 per week at the first 30 days to 2 and 1.4 and to 0.6 and 0.7 shoot per week at the 45 and 60 days of incubation respectively. However, the rate of explants subcultured for the second and third times increased from 1.4 and 0.93 shoot per week at the first 30 days to 2.6 and 2.7 shoots per week at the 45 days of incubation respectively. Therefore, to maintain a high rate, a system in which different incubations for each cycle of multiplication might be better than the commonly used multiple cycles and the fixed incubation period system. A system of a 30 days incubation during the first subculture followed by a 45 days incubation during the second and third and a 30 days long incubation during the fourth cycle is worth testing.

Initiation of pineapple shoot from leaf explants (Mercier *et al*., 2003) and etiolated nodes (Souza *et al*., 2003) occurred in 4 days and the shoot sprout within 14 days. Converting the shoot formation at different incubation periods (Table, 3.9) to percentage of shoot formation for each 15 days increment (Figure, 3.3) and to rate of shoot formation per week showed that overall subcultures, 35% of the shoot formation occurred in the first 30 days at a rate of 1.5 per week, 15%, in the following 15 days with a rate of 1.8 shoots per week, 10% over the next 15 days at a rate of 1.0 shoot per week while the majority of shoots (40%) at a rate of 3.8 shoots per week occurred in the last 15 days of the 75 days incubation (Figure, 3.3; Table, 3.9). The high percentage of shoot formation during the first 30 and the last 15 days of the 75 days incubation at all of the four subcultures indicated that neither the hormone concentration nor nutrient depletion and residual effect (accumulation of inhibitors) could explain the effect of incubation period and subcultures in the shoot formation pattern. Escalona *et al*. (1999) reported that the pineapple adjusted

the medium pH to equilibrium of 3.5 after 7 weeks and that coincided with the highest shoot rate. Frequent subcultures at 30 days did not allow the explants to fully adjust the medium and reached its highest potential for shoot formation. If it is so, the decline after the fourth subculture implies that the time required by the explants to adjust the medium pH increased at each subculture to exceed 75 days by the fourth subculture. However, the adjustment justification does not explain the high percentage of shoot formations observed over the first month of each subculture.

The different in shoot average between incubation periods and the two peaks could be related to the different number and maturity state of the pre-existing axillaries of the explants or different origins of the shoots. Shoots formed at the first 30 days originated from the pre-existing mature axillaries. Shoots appeared on the second peak came from partially matured preexisting axillaries that completed development during incubation and from axillaries of shoots formed during incubation (second axillaries). Miller and Drew (1990) attributed the formation of more shoots of papaya over longer incubation periods to second axillary buds. Harris and Mantell (1991) reported that a high percentage of shoots formed during 5 weeks incubation was of axillary origin and the rate was stable for three subcultures while shoots formed during 3 weeks were of adventitious origin and the rate decline over subcultures. Norton and Norton (1986) referred the stable rate of *Chaenomeles japonica* over nine subcultures to its ability of axillary shoot formation.

If a longer incubation increased the number of pre-existing axillaries per shoot while subculture slows down its maturity (full development), shoots formed during a short incubation would have few axillaries most of it partially developed. Hence, at each subculture a few shoots formed and rate decline over subcultures. In long incubation, the large number of axillaries per shoot and development of the partially developed, during incubation, compensate for the slow maturation effect imposed by subcultures and lead to

an increase in rate over the first three subcultures. The shoot formations average decreased sharply by the fourth subculture (Table, 3.9) and the usual explanation is residual effect of hormones (inhibitor accumulation). However, the pattern of shoot formation showed that the sharp decline was due to lack of shoot formation during the period between 30 and 60 days. If inhibitor accumulation was the cause, the decrease in shoot formation should appear during the first 30 days. The high percentage of shoot formation and rate per week during the first 30 and the last 15 days of the 75 days incubation and the persistence of this pattern at each of the four subcultures (Figure, 3.3) may indicate a lack of promoter rather than an accumulation of inhibitor were the cause of sharp decline by the fourth subculture.

The effect of subculture and incubation in shoot initiation and development process is not well understood. Besides being effected by external, the growth is controlled by internal factors. However, the decline in rate could be delayed for several subcultures. Destruction of tip chemically (Voyiatzi *et al*., 1995) and manually (Harris and Mantell, 1991) increased, respectively, rate of hybrid tea rose and Paeony over four subcultures. Vardja and Vardja (2001) reported that a decline in shoot formation of *Gerbera* observed by the fourth subculture could be delayed to the ninth subculture by reducing the hormone concentration at the fourth subculture and usage of hormone free medium by the seventh subculture. Using a hormone free medium by the fourth subculture was also suggested to restore the proliferation of *Arctostaphylos uva-ursi* (Kada *et al*., 1991). Selecting the proper hormone, TDZ rather than BAP, maintained a continuous increase in the rate of *Colocasia esculentia* (Chand *et al*., 1999) for five subcultures. Simultaneous decrease in rate and increase in hyperhydricity over subculture was observed in *Paeonia suffruticosa* (Bouza *et al*., 1994). In this study, extending the incubation period to75 days had reversed the decline in rate of shoot formation observed over the first three subcultures of a 30 days long incubation period. Longer incubation could be used as remedy to overcome the

Table 3.9 Effect of incubation periods and subcultures on the *in vitro* shoot formation and shoot length of Smooth cayenne pineapple shoot tip culture.

Incubation periods	Subcultures No				
(Days)	1	2	3	4	Average
A. Shoots per explants					
30	11 def	6 fg	4 g	4 g	6 C
45	13 cde	10 ef	12 cde	4 g	10 B
60	12 cde	12 cde	16 bcd	6 fg	12 B
75	17 bc	19 b	25 a	15 bcde	19 A
Average	14 A	13 A	12A	7 B	
B. Shoot length (mm)					
30	5 e	6 de	6 de	7 cd	6 C
45	7 cd	7 cd	7 cd	9 ab	8 B
60	8 bc	10a	8 bc	6 de	8 AB
75	9 ab	9 ab	10a	8 bc	9A
Average	7A	8A	8A	8A	

Data were means of 9 explants individually cultured on full strength agar solidified (7 g/l) MS medium supplemented with sucrose at 30 g/l and combination of BAP at 3.25 and IAA at 1.75 mg/l and adjusted to pH 5.7.
Means followed by different the letter were not significant at $p \leq 0.05$ according to Duncan Multiple Range Test.

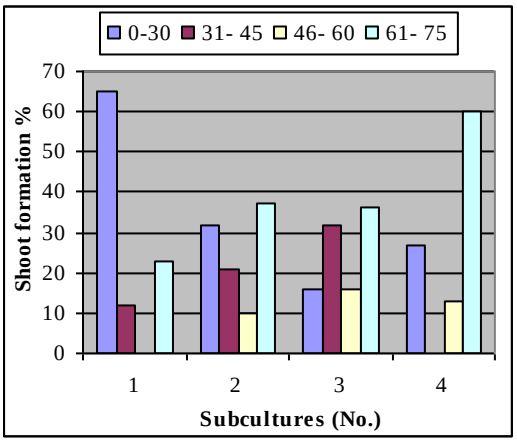


Figure (3.3) Effect of subcultures on the shoot formation percentage at different incubation periods compared to 75 days incubation

Table 3.10 Effect of incubation periods and subcultures on the total shoots production of Smooth cayenne pineapple shoot tip culture over consecutive subcultures

Incubation periods	**Subcultures**			
Days)	1	2	3	4
		Total shoots		
30	11 i	66 h	264 f	1056 e
45	13 i	130 g	1560 d	6240 c
60	12 i	144 g	2304 d	13824 b
75	17 i	323 f	8075 c	121125 a
		Total cost (RM)		
30	1.31	15.36	97.7	418.51
45	1.41	19.38	197.94	2350.73
60	1.51	19.27	232.39	3660.96
75	1.61	28.63	542.83	13437.36
		Cost per shoot (RM)		
30	0.07	0.15	0.25	0.27
45	0.07	0.10	0.09	0.25
60	0.08	0.09	0.07	0.18
75	0.07	0.06	0.05	0.08

The total shoots were estimated from using single shoot of Smooth cayenne.
Means followed by same letter were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range Test.
Shooth cayenne shoots were cultured at density of 1 shoot per glass jar containing 20 ml of full strength agar solidified (7 g/l) MS MS medium enriched with sucrose at 30 g/l, combination of BAP at 3.25 and IAA at .75 mg/l and adjusted to pH 5.7. Medium were dispensed at 20 ml per glass jar and autoclaved at 121 ^{0} C and 1.5 kg/l for 25 minutes and the cultures were incubated at constant temperature 25 ^{0} C and 16 hour of light provided by cool white florescence lamps for 75 days
RM. Ringgit Malaysia (1 USA $ = RM 3.25).
The calculated cost included only the variable items of cost (Jars, MS, Sucrose, Agar, labor wages and electricity cost for operating autoclave, laminar and incubation room).

Table (3.11). Cost items and estimated budget required for multiplication of Smooth cayenne pineapple for four subcultures

Cost items		Subcultures				
		1	2	3	4	Total
		Multiplication cost (RM)				
Jars		0.5000	8.000	153.000	3876.000	3876.00
Medium	**MS**	0.1600	2.720	51.680	1292.000	1346.560
	Agar	0.0773	1.314	24.961	624.036	650.388
	Sucrose	0.0528	0.898	17.054	426.360	444.365
Hormones	BAP	0.0052	0.088	1.680	41.990	43.763
	IAA	0.0005	0.008	0.147	3.674	3.829
Electricity	Incubation	0.5000	8.500	161.500	4037.500	4208.000
	Autoclaving	0.0750	1.275	24.225	605.625	631.200
	Laminar	0.0417	0.708	13.458	336.458	350.667
Labor	Jar cleaning	0.0000	0.111	1.889	35.889	37.889
	Mprep	0.0333	0.567	10.767	269.167	280.533
	Culturing	0.1667	2.833	53.833	1345.833	1402.666
Total per subculture (+ Jars)		1.612	27.022	514.195	12894.530	13437.359
Cumulative total (+ Jars)		1.612	28.634	542.829	13437.359	
Total per subculture (- Jars)		1.112	19.022	361.195	9018.533	9399.862
Cumulative total (- Jars)		1.112	20.134	381.329	9399.862	
Total shoots		17	323	8075	**121125**	
Cost per shoot (include jars)		0.095	0.089	0.067	0.111	0.111
Cost per shoot (minus jars)		0.065	0.062	0.047	0.078	0.078

The total shoots were estimated from using single shoot of Smooth cayenne.
Shooth cayenne shoots were cultured at density of 1 shoot per glass jar containing 20 ml of full strength agar solidified (7 g/l) MS medium enriched with sucrose at 30 g/l, combination of BAP at 3.25 and IAA at .75 mg/l and adjusted to pH 5.7. Medium were dispensed at 20 ml per glass jar and autoclaved at 121 ^{0} C and 1.5 kg/ cm^{2} for 25 minutes and the cultures were incubated at constant temperature 25 ^{0} C and 16 hour of light provided by cool white florescence lamps for 75 days
RM. Ringgit Malaysia (1 USA $ = RM 3.25).
The calculated cost included only the variable items of cost (Jars, MS, Sucrose, Agar, labor wages and electricity cost for operating aurtclave, laminar and incubation room).

decline in shoot formation rate after several subcultures and restore the proliferation capacity of pineapple shoots.

3.2.5. Effect of benzylaminopurine (BAP) concentrations on the multiplication and growth of Smooth cayenne pineapple

Table 3.12 showed that the shoot formation increased by one shoot in response to each 0.25 mg/l increase in the concentration of BAP up to 1.0 mg/l at almost steady state (one shoot/ one unit) and then then a sudden surge in shoot formation took place. Increasing the concentration by 0.25 mg/l, from 1.0 to 1.25 mg/l resulted in formation of 4 new shoots (from 6 to 10 shoots/ explant). Then, the rate of shoot formation remained almost stable around 11 shoots per explant for the four consecutive increments of 0.25 mg/l. After that a sharp decline occurred from 12 to 8 shoots / explant in response to just a minute increase of concentration by 0.25 mg/l from 2.25 to 2.50 mg/l. The rate remained stable at 8 shoots per explant for four consecutive increases in BAP concentration by 0.25 mg/l at each time till the concentration reached 3.25 mg/l. After that increase of concentration by 0.25 mg/l from 3.25 to 3.5 mg/l caused a sudden reverse in the shoot pattern and a second surge of shoot formation (from 7 to 12 shoots per explant) occurred. However, the potential for shoot formation the explants had just gained was lost upon raising the concentration to 3.75 mg/l. There were two sharp increases in shoot formation. The first occurred over a wide range of BAP concentrations. Compared to the shoot formation at 1.0 mg/l, increasing the BAP concentration to 1.25, 1.5, 1.75, 2.0 and to 2.25 mg/l caused abrupt increase in shoots formation from 6 to 10, 11, 11 and to 12 shoots respectively. On contrary, the second pulse of shoot formation was very specific and took place when the concentration increased from 3.25 to 3.5 mg/l. Similarly, there were two sudden declines. One took place as concentration increased from 2.25 mg/l to 2.5 mg/l or 2.75, 3.0, 3.25 mg/l. A second sudden lose of shoot formation capability occurred as the BAP

concentration increased from 3.5 to 3.75 mg/l. In general, there were no significant different between BAP concentrations of 1.75, 2.0, 2.25 and 3.5 mg//l, each induced the highest shoot formation (12 shoots/ explant). No significant differences between BAP at 2.5, 2.75, 3.0, and 3.75 mg/L each resulted in intermediate proliferation of 8 shoots per explan. At any BAP concentrations below 1.0 mg/l, the shoot formation was less than 6 shoots per explants.

The effect of different BAP concentrations on average shoot length and average weight per explant followed a similar pattern. The tallest (25 mm) and heaviest (0.67 g) shoots obtained in hormone free and in medium enriched with BAP at 0.25 mg/l and the shortest (7 mm) and lightest (0.13 g) shoots at 1.25 mg/l. The length and weight decreased as concentration increased from 0.25 to 1.25 mg/l. BAP concentration ranged from a minimum of 0.5 mg/l in case of average weight of shoots and 1.25 mg/l in case of average length of shoot, up to 3.75 mg/l did not cause any significant different in shoot fresh weigh and length per explant (Table, 3 12). Both the shoot weight and shoot length average have negative correlation (r = - 83 and - 76 respectively) with shoot formation (Table, 3.13). The pattern of the total weight and total length of shoots were similar, but opposite to the pattern of average weight and length of shoots. Both the total length and weight were not affected by BAP concentration ($p < 0.2135$ and $p < 0.2496$), correlated positively with shoot formation (r = 93 and 80) and with each other (r = 85) and negatively with average shoot length (r = - 66 and -58) and with average shoot weight (r = - 66 and 45). The lowest total length (37 mm) and total weight (0.99 g) was obtained on hormone free medium and the largest total length (109 mm) and heaviest total weight (2.61 g) on medium containing 3.5 gm/l of BAP. Each of the three processes of shoot formation, elongation and weight responded differently to presence of BAP. The total weight and length were not significantly affected by inclusion and omission of BAP while best average weight and

length obtained in hormone free medium. The best shoot formation, on the other hand, occurred in 4 different concentrations of BAP. BAP at 3.5 mg/l would be a best choice for shoot formation, total weight and total length. A dual medium purpose (propagules and biomass)

Several researchers demonstrated the suitability of singly applied BAP for pineapple culture. However, either few levels of BAP were tested or the increment was too large. A possibility that one of the untested concentrations could be better than the reported concentration ranges does exist. The proliferation rate of 12 shoots obtained in response to 1.75, 2.0, 2.25 and 3.5 mg/l (Table, 3.12) is two times higher than that reported by Bhatia and Ashwath (2002) and about equal to that reported by Sripaoraya *et al.* (2003) in response to BAP at 2.0 mg/l and higher than the 9, 7 and 3 shoots reported respectively by Be and Debergh (2006), Aydieh *et al.* (2000) and Zepeda and Sagawa (1981) in response to BAP at 1.0 mg/l, 10 shoots (Firozabady and Gutterson, 2003) in response to 3.0 mg/l and 3 shoots (Almeida *et al*., 2002) in response to 1.5 mg/l of BAP. The results did not support the use of BAP at 2.5 mg/l (Ko *et al*., 2006: Smith *et al.*, 2002). Using the same approach of testing of broad concentration range had enabled Sujatha and Reddy (1998) to select treatment that increased castor proliferation rate five times higher than previously reported.

The pineapple tissue culture is usually done for propagation purpose and the best treatment judged by the rate of shoot formation. Other parameters such as total and average fresh weight were rarely reported. Few reported the shoot length (Soneji *et al.*, 2002a; Rahman *et al*., 2001; Aydieh *et al*., 2000; Khatun *et al*., 1997) and shoots weight (Be and Debergh, 2006; Escalona *et al*., 1999). As the goal was propagules production, neglecting of weight are understandable. However, since there is possibility for extraction of bromelain for medical purpose and use of biomass for animal feeding reporting of weight

would be more important than any other parameters. Perez *et al.* (2003) obtained higher fresh weight, protein content and protease activity on response to BAP at 0.5 mg/l. Be and Debergh (2006) reported that although BAP at 1.0 mg/l and combination of BAP at 1.0 plus IBA at 0.5 mg/l induced equal number of shoots, the second treatment doubled the total fresh weight/ culture.

The shoot formation pattern (Table, 3.12) showed that the response of explant to an increase in BAP level depended on the previous concentration the explants were subjected to and the number of concentration increments included in the comparison. Over concentration ranges of 0.25 to 1.0 mg/l each increase of BAP concentration by 0.25 mg/l resulted in formation of one more shoot, a clear linear relationship. Aydieh *et al*. (2000) and Be and Debergh (2006) reported similar findings. However, an increase by 0.25 mg/l after the concentration reached 1.0 mg/l resulted in sudden pulse of shoot formation. Four instead of one shoots were formed in response to 0.25 mg/l increases in concentration from 1.0 to 1.25 mg/l. The explants treated with 1.25 mg/l of BAP almost remained unresponsive to an increase in BAP by 0.25 mg/l for three consecutive times. Shoots formation rate over concentration range of 1.25 to 2.25 mg/l were almost equal (11 shoots per explant). However, when the concentration reached 2.25 mg/l, increasing of concentration by 0.25 mg/l resulted in drastic sudden drop of shoot formation. Just 0.25 mg/l increase in concentration made the explants lost 30 % of its shoot formation capability. The shoot number dropped from12 shoots at 2.25 mg/l to 8 shoots at 2.5 mg/l. Then the explants remained unresponsive for four consecutive increases of 0.25 before it regain a sudden sharp increase in shoot formation capability. Increasing of concentration by 0.25 mg/l from 3.25 to 3.5 increased the shoot formation by 30 % where the shoot number increased from 7 to 12 shoots. However, contrary, to the first sudden increase obtained between 1.0 and 1.25 mg/l, this second sudden increase did not persist. Further

increase by 0.25 returned the explants to its previous shoot formation capacity of 8 shoots / explant. It is quite interesting that the sudden increase and decrease in shoot formation followed symmetrical trend. Each were of equal magnitude resulting in gaining or losing capability to form 4 to 5 shoots than the proceeding concentration level. Each occurred after 4 consecutive addition of 0.25 and the loss or gain of proliferation capacity persisted for the next 4 increments. On contrary, the second pulse of increase lost immediately afterward.

Construction of functional relation between concentrations and the parameters of interest and estimation of the degree of association between the different parameters are very important to understand the causes and results of physiological events. But, it could not be done if the concentrations range is just few levels and when unequally distened concentrations and large increment was used as it is the case in all reported studies. Correlation coefficient (r = 0.63) indicated that the shoot formation increased as BAP concentration increased and regression analysis showed that 71 % of the difference in shoots formation was caused by difference in concentrations of BAP. Table (3.13) showed that the shoot length and weight correlated positively with each other (r = 0.82) and negatively with BAP concentration (r = - 0.73 and - 0.54 respectively). Shoot formation correlated negatively with average length (r = - 0.83) and weight (r = - 0.76) and positively with BAP concentration (r = 0.63). The shoot elongation and weight responded only to low concentrations and narrow range (0.25 to 1.0 mg/l) of BAP. Any concentration above 1.0 and up to 3.75 mg/l had no significant effect on either average length or weight of shoots but induced significant effect on shoot formation (Table, 3.12). Regression coefficient indicated that 93, 87 and 71 % of different in average length, weight and shoot number were due to difference in BAP concentration (Table, 3.13). These correlation results indicated that the shoot elongation and weight proceeded simultaneously but at alternative

time cycle with shoot formation process. The high correlation and regression coefficient indicated that within BAP concentration range of 0.25 to 3.75 mg/l the shoot formation and shoot length and weight could be estimated as a function of BAP (Table, 3.13). Deora and Shekhawat (1995) suggested similar model for *in vitro* shoot formation of *Capparis deidua.*

Although total weight and length of shoots obtained from a single explant is a product of multiplying of average shoot number by average shoot weight and length which both were significantly effected by BAP, the differences in total weight ($p < 0.2496$) and total length ($p < 0.2135$) were not significant. This might be related to the value and nature of BAP effect on these processes. Shoots formation were positively affected while average length and weight of shoots were negatively affected by presence of BAP. In addition, the average length and weight of shoots correlate positively with each other but negatively with amount of shoot production. Such relation might neutralize the overall effect of BAP on total weight and total length of shoots per explant. Existing of positive correlation between shoot numbers, total weight, total length (Table, 3.13) indicated that a system for optimum production of both propagules and biomass is a possible using singly applied BAP. In addition, the high correlation between number and total weight of shoots ($r = 0.80$) indicated that rather than individual counting, the shoot number could possibly estimation as function of total weight according to the model presented (Table, 3.13). Firoozabady and Gutterson (2003) counted the number of shoot in specific unit of weight and used that ratio for estimation of shoot number from a total weight obtained in bioreactor system. Salehi and Khosh-Khui (1997) suggested a model by which the shoot length after one week in culture could be used for estimation of the number of shoot that miniature rose would produce after four weeks of incubation.

Table 3.12. Effect of BAP concentrations on shoot formation (No.), and average (x) and total (T) of shoot length and shoot weight per explant of Smooth cayenne pineapple cultured on agar solidified full strength MS medium for 60 days

BAP (mg/L	Parameters				
		Average per explant		Total per explant [NS]	
	Shoot No	Shoot leng (x)	Shoot wt. (x)	Shoot leng (T)	Shoot wt. (T)
0.00	2 f	20 a	0.60 a	0.99	37
0.25	3 ef	14 b	0.67 a	0.99	40
0.50	4 def	14 b	0.27 b	1.07	55
0.75	5 cdef	12 bc	0.24 b	1.12	57
1.00	6 bcde	11 bc	0.22 b	1.34	67
1.25	10 abc	7 c	0.13 b	1.32	70
1.50	9 abcd	9 bc	0.13 b	1.20	78
1.75	11 ab	8 c	0.15 b	1.54	84
2.00	11 ab	8 c	0.16 b	1.89	94
2.25	12 a	8 c	0.18 b	2.20	96
2.50	8 abcde	8 c	0.18 b	1.49	64
2.75	8 abcde	9 bc	0.24 b	1.55	64
3.00	8 abcde	8 c	0.21 b	1.70	68
3.25	7 abcde	8 c	0.24 b	1.70	56
3.50	12 a	9 bc	0.22 b	2.62	109
3.75	8 abcde	8 c	0.19 b	1.57	69

Data were means of 9 shoots that were individually cultured in 20 ml of agar solidified (7 g/l) full strength MS medium containing 30 g/l of sucrose, BAP at different concentrations, adjusted to pH 5.7 after 60 days incubation.

NS. No significant effect on total weight ($p \leq 0.2496$) and total length ($p \leq 0.2135$)

Table 3.13. Correlation and regression coefficient between BAP concentrations and growth parameters of Smooth cayenne pineapple cultured on agar solidified MS medium for 60 days

BAP	**Growth parameters**				
Concentration	No	Lx	Wx	WT	LT
A. Correlation					
r	0.63	-0.73	-0.54	0.75	0.55
P	0.0088**	0.0013**	0.03*	0.0008**	0.0275*
Total weight					
r	(0.80)				
P	0.0002**				

Shoot number = -10.609 + 18.020 (Total weight) - 3.613 (Total weight)^2

B. Regression

Equations	**R^2**	**P**
Shoot number = 1.561 + 6.962 (BAP conc) - 1.417 (BAP conc.)^2	71	0.0027
Shoot length = 19.126 - 14.178 (BAP conc) + 5.752 (BAP conc.)^2	93	0.0001
Shoot weight = 0.674 - 0.801(BAP conc.) + 0.373 (BAP conc.)^2 - 0.052 (BAP conc.)^3	87	0.0009

No (number of shoots); Lx (average length of shoot); LT (total length of shoots); Wx (average fresh weight of shoot); WT (total fresh weight of shoots)

Most importantly, the success of chemical and histological studies depends on proper timing of these studies. The pattern of shoot formation over wide concentration range and small increments would facilitate designing proper experiment and selection of the most proper concentrations for monitoring the histological and chemical changes that lead to shoot formation. It seemed that there is certain shoot promoter and shoot inhibitors that could be formed in response to presence of specific levels of BAP. Both promoters and inhibitors could be formed over wide range of concentrations. Promoter formed most likely in response to 1.25 and 3.5 mg/l of BAP and the inhibitor in response to 2.5 and 3.75 mg/l of BAP. Comparing of histological results and chemical analysis of explants and media of the explants cultured in medium enriched with 1.25 and 3.5 mg/l to that of the explants cultured in medium enriched with 2.5 and 3.75 mg/l would help in identifying the compounds that caused the sudden increase or decline in the shoot formation. Confirmation would require that the compound isolated and tested for its expected effect. Chemical analysis of agar-solidified medium was successfully used for identification of anti-hyperhydricity factors and facilitates commercial micropropagation of radiata pine (Nairn *et al*., 1995).

3.3.6. Effect of density of separated and cluster shoots on the shoot formation rate per shoot, total shoots per liter of medium and cost per shoot of Smooth cayenne pineapple

The average shoot formation and shoot length per separated shoots and cluster of shoots and the estimated total shoots per liter of medium (Table, 3. 14) showed that increasing the number of shoots per cluster (cluster size) from 2 to 4 shoots reduced the shoot formation capability of each single shoot of the cluster from 7 to 4 shoots and increased the shoot length from 13 to 16 mm, but did not induce any significant different in the estimated total of shoot production per one liter of medium. The total shoots per liter

using cluster of 2, 3 and 4 shoots were 700, 750 and 800 shoots respectively. However, increasing the cluster size (number of shoots per cluster) from 1 to 4 shoots increased the cost per shoot from 13 to 20 Malaysian cents per shoot. Similar, the shoot formation capability of the separated shoot decreased from 11 to 7 shoots as the density increased from 1 to 4 shoots per culture. The shoot length increased from 12 to 15 mm as the density increased from 1 to 2 shoots per culture. However, the density lost its effect on shoot length when 3 and 4 shoots were used per culture. Equal shoot lengths of about 14 mm were obtained at either density. Contrary to shoots cluster in which the estimated total shoots per liter were not effected, increasing the density of the separated shoots per culture to 3 and 4 shoots resulted in significantly higher total per liter (1200 and 1400 shoots) than using of 1 and 2 (550 and 800 shoots) shoots per culture and reduced the cost from 13 and 12 cents at density of 1 and 2 shoots to 10 cents at density of 3 and 4 shoots. The total shoots produced using cluster of 3 and 4 shoots (700 and 750 shoots) was not significantly different from that at density of 1 (550 shoots) and 2 (800 shoots) separated shoots but was two times less and two times more expensive than that at density of 3 (1200 shoots at 10 cents) and 4 (1400 shoots at 10 cents) separated shoots per culture.

In most pineapple *in vitro* multiplication studies, the attention was focused on optimization of hormones and medium states and the treatments assessment were based on shoot formation rate per explant. Treatment with highest shoot formation per explant were considered optimal and suggested for commercial production of propagules. Both hormone and medium state are very important, but other factor as well could independently or through interaction with hormone and medium state affect the rate of shoot formation. In addition, higher shoot formation per single explant is not the best indicator for commercial production. Total shoot production, total cost and cost per single shoot rather than the rate per single explant are the most practical and essential parameters of

commerciality. Table 3.14 showed that at any density, the separated shoots resulted in higher shoot formation rate than cluster of shoots. In both cases, assessment based on shoot formation per explant was not enough for selection of best treatment. Using of shoot formation per single shoot as parameter for treatment assessment, one separated shoot and one cluster of two shoots per culture would be recommended over higher shoot density and larger cluster size. On the contrary, using the estimated total of shoot production per one liter of medium as parameter of assessment indicated that separated shoots at density of 3 and 4 shoots per culture increased the total by two to three times and reduced the cost per shoot by two times compared to density of 1 shoot per culture. Similar, increasing the cluster size (shoots per cluster) reduced the average of shoot formation per each shoot of the cluster, but had no effect when estimated total shoots per one liter of medium was compared. It is interesting that whether the explants were separated shoots or cluster of shoots, higher explants density decreased the shoots formation per single shoot. However, while increasing the density of separated shoots increased the total shoots per liter of medium, the estimated total of shoots of the different cluster sizes (2, 3 and 4 shoots/ cluster) were not significantly different. The different shoot formation rate and total shoots per liter using separated and cluster shoots indicated that the recommended treatment for one explant type could not be expected to be optimal for the other and the importance of using more than one parameter for treatment assessment. The rate of shoot formation per explant, the total shoots production as well as the cost per single shoots was significantly affected by both of the explants density and size of the shoot clusters and the cost per single shoots could be reduced to as low as 10 cents by using of 3 and 4 separated per culture (Table, 3.14).

Separated shoots at density of 1 (Sripaoraya *et al*., 2003; Devi *et al*., 1997), 2 (Be and Debergh, 2006; Soneji *et al*., 2002a), 3 (Hamad and Taha, 2003) and 4 shoot per culture

(Perez *et al*., 2009) and cluster of 1 to 3 shoots at density of 1 cluster per culture on conventional (Firoozabady and Gutterson, 2003; Escalona *et al*., 1999) and 50 clusters on bioreactor system (Escalona *et al*., 1999) and shoot formation rate range of 3 to 25 shoots per explant on conventional and 60 shoots per cluster on bioreactor system were reported. However, neither the effect of different explants density nor the effect of different size of clusters on the shoot formation rate of pineapple and the expected total of shoots was compared in the previous studies. The shoot formation rate obtained in this study at density of 1 and 2 separated shoots per culture were within the rate range previously reported for Smooth cayenne on solid full strength MS medium. The little different on the rate could be attributed to different hormone treatments and incubation periods. Although easy to test and simply to apply, comparing the effect of explants density and explants manipulation on the *in vitro* multiplication of pineapple was totally ignored in the previous study of pineapple. Total shoots per liter, on the other hand, is more practical and essential for better management and planning of commercial micropropagation than the rate of shoot formation per explant.

Cost of production was always stressed as the main obstacle of micropropagation. Nevertheless, the cost items such as medium volume and medium use efficiency (total shoot per liter), autoclaving and laminar operation time, labor working hours and shelving space was not considered during assessment of different *in vitro* multiplication treatments. All of these cost items are related to and could be manage through selection of the explant types and density of explant per culture. Yet the effect of these two factors on the rate of shoot formation and its relation to the different cost items were not reported. Arinaitwe *et al.* (2000) used the cost of one liter of medium as basis to compare the cost effectiveness of different cytokinin on banana shoot formation. Using the same approach, Goel *et al.* (2007) reported that cost per shoot of *Rauwolfia serpentina* in semi solid was 0.126 Rs and

could be reduced to 0.004 Rs (Indian currency) using glass bead and Daurala sugar. Our estimation of cost per pineapple shoot included all cost items of multiplication (medium, culture tubes, electricity cost for operating autoclave, laminar and incubation room and the labor wages). Using of the shoot cluster resulted in lower rate per single shoot, lower total shoots per liter of medium and higher cost per shoot than separated shoots. However, longitudinal segmentation of the multiple shoots into cluster of shoots is much simpler and easier than separation into individual shoots and hence it would take shorter laminar operating time (electricity cost) during subculturing into fresh medium (labor cost). Random cutting into shoot clusters deserve further investigations to improve the shoot formation capacity per explant and total shoots per liter of medium. The lower shoot formation per shoot of cluster may be due to domination effect of one shoot of the cluster over the others. In this study both separated and cluster shoots were treated by same hormone and equal concentration (BAP at 2.25 mg l^{-1}) at fixed incubation period. Using other concentration of BAP, other type of cytokinin and different incubation periods may overcome the domination effect of one shoot over the other and improve the proliferation capacity of the cluster. Using bioreactor system, Escalona, *et al*. (1999) reported that the effect of clusters density per bioreactor on the total shoot production depended on the incubation period. At 4 weeks incubation, 50 clusters of shoot per 10 liters bioreactor produced higher total of shoots than density of 25 clusters of shoots. However, if the incubation length increased to 8 weeks, density of 25 resulted in higher total. Investigation the effect of clusters consisted of more than 4 shoots and separated shoots at density higher than 4 separated shoots per culture on the shoot formation and cost on conventional micropropagation system could reduce the cost to lower than the estimated-10 cents per shoot obtained in this study.

Table (3. 14). Effect of shoot density (shoots per culture) and shoot manipulation (separated and cluster) on the *in vitro* shoot formation per single shoot, shoot length (mm), total shoot per one liter of medium and expected cost per shoot of Smooth cayenne pineapple.

Shoots / culture	**Shoots manipulation**							
(No.)	Clustered	Separated	Clustered	Separated	Clustered	Separated	Clustered	Separated
	Shoot / explant		Shoot length (mm)		Total shoots / l		Cost / shoot	
1	11 a	11a	12 b	12 b	550 b	550 b	0.17	0.17
2	7 bc	8 ab	13 ab	15 ab	700 b	800 b	0.19	0.14
3	5 bc	8 ab	13 ab	13 ab	750 b	1200 a	0.21	0.11
4	4 c	7 bc	16 a	14.7 ab	800 b	1400 a	0.22	0.11
Average	6.75 B	8.5 A	13.5 NS	13.7 NS	700 B	987.5 A		

Means of the same column followed by the same small letters and the average followed by same capital letters were not significantly different at $p \leq 0.05$ as tested by Duncan Multiple Range Test.
RM Ringgit Malaysia (1 USA $ = RM 3.25).
The calculated cost included only the variable items of cost (MS, agar, sucrose, hormones, electricity cost of autoclave, laminar operation and incubation room and the wages of labor).

Which explant types and explant density should be used would depend on the availability of culture stocks, amount and time of propagules delivery and the available budget. If the mother plants were limited and few stock cultures were successfully established as it is usual during the establishment stage, subculturing of shoots at density of one shoot per culture is essential to take the advantage of high shoot formation per single shoot. However, by the second cycle of multiplication stage, more shoots would be available, using of separated shoots at higher density (3 and 4 shoots) per culture, in addition to production of higher shoots total per liter of medium, would reduce the cost per shoot (less jars, medium, shelving space and working time). In conclusion, although optimization of hormones and medium states are very important for proliferation studies, adoption of a shoot multiplication system for commercial application require using of proper explants density and explants manipulation. Compared to explant density of one shoot per culture, the cost of the *in vitro* shoot production of Smooth cayenne pineapple could be reduced to half by culturing at density of 3 separated shoots per culture. In addition to the rate of shoot formation per explant, estimated total per unit of medium volume should be taken in consideration. Total shoots per liter and total at each cycle of multiplication is very essential and more practical for estimation of cost and for better management and planning of commercial micropropagation than the rate per explant.

3.2.7. Effect of hormone types, concentrations and subcultures on the *in vitro* shoot formation rate per explant, total shoots production and cost of *in vitro* multiplication of Moris pineapple

ANOVA analysis revealed that the process of *in vitro* shoot formation and growth was affected by the types and concentrations of the hormones and the shoot proliferation capacity depended on how many times the shoot was subcultured. Shoot formation and total shoots production were significantly affected by independent influence of hormone concentrations, types and subcultures and interaction between any two and all of the three factors. Shoot length, on the other hand, was affected by all factors independently and by interaction of any two but the collective interaction of the three factors was not significant ($p \leq 0.4667$). Irrespective of hormone type, the shoot formation increased over the first two subcultures and slowed down by the third (Figure, 3.4A). However, while the shoot lengths of the explants treated with NAA, IAA and KN were increasing, the shoot lengths in response to BAP were significantly decreasing over subculturing (Figure, 3.4B). Repeated subculturing in medium enriched with BAP and IAA for three times resulted in the highest total of shoots from a single explant (98 and 79 shoots) while the lowest total (30 shoots) were obtained in medium enriched with KN (Figure, 3.4C). Intermediate total of 59 shoots obtained in medium enriched with NAA.

Table 3.15 showed, on the other hand, that the explants capability for shoot formation not only varied according to hormone types but also at different concentrations of the same

hormone and over subcultures. The highest shoot formation average over three subcultures (6 shoots per explant) obtained in media enriched with BAP at 2.0, 3.0 and 3.5 mg/l; NAA at 2.0 and 5.0 mg/l and IAA at 2.0, 3.0, 3.5 and 4.0 mg/l and the lowest average (2 shoots per explant) obtained on media containing KN at 1.0 mg/l and IAA at 0.5 mg/l. Compared to hormone free medium, 8 of 10 BAP, NAA and IAA concentrations resulted in more shoots than hormone free and equal rate obtained in the other two concentrations. On contrary, only two concentrations of KN resulted in more shoots than hormone free while the shoot formation at eight concentrations was not significantly different from hormone free. KN is definitely not a suitable hormone for proliferation of Moris pineapple.

Generally, the shoot formation increased as BAP increased up to 3.5 mg/l and decline afterwards. According to their effect on the average and total shoot formation over three subcultures (Table, 3.15), the BAP concentrations could be grouped into four groups. The first group consisted of two levels (4.0 and 5.0 mg/l) resulted in equal rate (3 shoots) but less total shoots (12 and 16 shoots) than hormone free medium. The second groups consisted of two levels (1.0 and 4.5 mg/l), both resulted in equal rate (4 shoots) but the total shoots of the first concentration (60 shoots) was two times the second (30 shoots). The third group consisted of two levels (1.5 and 2.5 mg/l) and both resulted in equal rate (5 shoots) and total shoots (120 shoots). The fourth groups consisted of three levels (2.0, 3.0 and 3.5 mg/l) and were the most effective concentrations of BAP. They resulted in equal rate (6 shoots) but different total shoots (160, 210 and 180 shoots respectively). Similar,

the concentrations of KN, NAA and IAA could also be grouped into 4 groups. The best concentration of KN was 0.5 mg/l. It resulted in average of 5 and total of 90 shoots per three subcultures. KN at 1.5 and 4.0 mg/l resulted in equal average (4 shoots per explant) and statistically equal total shoots (48 and 42 shoots respectively). KN at 2.0, 2.5 and 3.5 mg/l and KN at 1.5 and 4.5 mg/l and KN at 5.0 mg/l were least effective KN concentrations and all resulted in equal rate (3 shoots) as hormone free medium. But while the first group resulted in total shoots as hormone free medium (27 shoots), the second group resulted in lower total shoots (12 shoots) and the third group resulted in higher total (32 shoots) than that of hormone free medium. The highest rate (6 shoots) in response to NAA obtained at three concentrations (0.5, 2.0 and 5.0 mg/l) but each concentration resulted in different total of shoots, (88, 160 and 135 shoots respectively). NAA at 1.0 and 4.0 mg/l resulted in equal rate (5 shoots) and equal total (42 shoots). NAA at 3.0 mg/l resulted in higher shoot formation rate (4 shoots per explant) than but equal total as that of hormone free medium (both 28 shoots) while NAA at 3.5 mg/l resulted in equal rate as (3 shoots) but lower total shoots (16 shoots) than hormone free medium. The lowest total shoots (4 shoots) and the most stunted shoots (4 mm) of all combinations of hormone types and concentrations obtained in medium contained IAA at 0.5 mg/l. All IAA concentrations except at 0.5 mg/l resulted in higher rate and total than hormone free medium. Four IAA concentrations (2.0, 3.0, 3.5 and 4.0 mg/l) resulted in equal average (6 shoots per explant) but different total shoots (72, 200, 104 and 60 shoots respectively). Two (1.5 and 5.0 mg/l)

and three concentrations (1.0, 2.5 and 4.5 mg/l) resulted in formation of 5 and 4 shoots per explants respectively, but the total shoots of the first group was more than 100 shoots and that of the second was less than 73 shoots.

At each subculture there were different optimal concentrations of the same hormone as well as different optimal type of hormone (Table, 3.15). The highest shoot formation in response to BAP at the first subculture (7 shoots) obtained in medium containing BAP at 3.0 mg/l while the highest at the second (9 shoots) and third (6 shoots) obtained in medium enriched with BAP at 3.5 and 4.0 mg/l respectively. In case of KN, the highest shoot formation during the first (3 shoots) and second subculture (10 shoots) obtained in medium enriched with KN at 0.5 mg/l while the highest shoot formation during the third subculture (4 shoots) obtained in medium enriched with KN at 2.5, 3.0, 4.5 and 5.0 mg/l. Similar, different NAA concentrations, 2.0, 0.5 and 2.5 mg/l, resulted in the highest shoot formation (4, 11 and 7 shoots) during first, second and third subculture respectively. For IAA, starting with highest concentration of 5.0 mg/l during the first and decreasing the concentration to 3.5 and 1.0 mg/l during the second and third subcultures resulted in the highest shoot formation (5, 13 and 5 shoots) than other IAA concentrations. Generally, of all treatments, the highest shoot formation during the first subculture (7 shoots) obtained in medium with BAP at 3.0 mg/l. The highest at the second (13 shoots) and the third (7 shoots) subculture obtained in medium enriched with IAA at 3.5 and NAA at 2.5 mg/l respectively (Table, 3. 15).

Average overall hormone types and concentrations showed that the shoot formation capability increased over subcultures from average of 2 shoots at first to 5 and 6 shoots per explant at the second and the third subculture. However, individual comparison of all treatments (Table, 3.15) showed that this trend was only true in few cases (BAP at 4.0 and 5.0 mg/l; KN and NAA at 2.5 and 3.0 mg/l and IAA at 1.0 mg/l). In medium enriched with BAP at 1.5, 3.0 and 4.5 mg/l; KN at 4.5 and 5.0 mg/l and NAA at 3.5 mg/l. the rate increased over the first two subcultures but remained stable afterwards. In most combinations of hormones and concentrations the shoot formation increased over the first two subcultures and decreased by the third (Table, 3.15; Figure, 3.4A). On contrary, in two cases, media enriched with BAP at 3.0 mg/l and IAA at 4.5 mg/l the highest shoot formation obtained during the first subculture and declined afterward. None of the treatments except hormone free medium maintained the rate stable over subcultures. Decline in rate of pineapple shoot formation was reported after the second (Fitchet, 1990a), fourth (Singha and Manual, 2000) and seventh (Kofi and Adachi, 1993). The cause of decline is not clearly understood. However, hormone types and concentrations are two of several other factors that could be the cause of such decline in shoot formation. At fixed concentration of BAP, Moncalean, *et al*. (2000) found that the proliferation pattern of kiwi apical shoot over three subcultures varied according to the length of exposure time to BAP. Explants exposed to BAP for 30 minutes, 8 hours and 4 days showed stable rate of shoot

formation over subcultures. However, the rate over subcultures decreased when the explants were exposed for only 1 day and continuously for 35 days.

It is obvious that the total shoots production would be increased over subcultures. But at equal subcultures the total shoots varied markedly among the different hormone types and within the different concentrations of the same hormone (Table, 3.15). The highest total at the first (7 shoots), second (42 shoots) and the third subcultures (210 shoots) obtained from explants treated with BAP at 3.0 mg/l. By the second subculture, the proliferation capacity of explants cultured on medium enriched with BAP at 2.0, and 3.0 mg/l and with IAA at 3.0 and 5.0 was equal (40- 42 shoots). But, by the third subculture, explants treated with BAP at 2.0 and IAA at 5.0 mg/l lost its shoot formation capability and produced fewer total (160 and 120 shoot respectively) while those treated with BAP at 3.0 and IAA at 3.0 mg/l maintained their proliferation capacity resulting on 210 and 200 shoots respectively. The differences in total shoots among the different treatments were so wide. It ranged from a lowest of just 5 shoots obtained from explants treated with IAA at 0.5 mg/l to a highest of 210 shoots from explants treated with BAP at 3.0 mg/l. By the third subculture, treatment such as BAP at 2.0, 3.0 and 3.5, NAA at 0.5, 2.0 and 5.0 and IAA at 2.0, 3.0 and 4.0 mg/l. which resulted in equal shoot average overall subcultures (6 shoots per explants) resulted in different total of 160, 210, 180, 88, 160 135, 72, 200, 104 and 60 shoots respectively). BAP is the most used hormone for *in vitro* shoot proliferation. NAA and IAA are usually applied in combination with cytokinine merely to enhance the

cytokine effect on shoot formation. Generally, NAA and IAA are considered as rooting hormones and applied singly and in combination with IBA for root induction. However, Table (3.15) showed that provided proper concentration was used either of BAP, NAA or IAA could be used for induction of shoot formation of Moris pineapple. Overall subcultures, the highest formation of 6 shoots obtained at 3 levels of BAP (2.0, 3.0 and 3.5 mg/l) 3 levels of NAA (0.5, 2.0 and 5.0 mg/l) and 4 levels of IAA (2.0, 3.0, 3.5 and 4.0 mg/l). Singly applied NAA and IAA particularly during the third subculture did induce rooting of Moris pineapple (data not shown) but on the same time multiple shoot formation occurred simultaneously. A treatment with dual purpose of simultaneous rooting and multiplication is advantageous at least in term of production cost. Although multiplication of shoots is always induced in medium enriched with BAP alone and in combination with auxins, single application of NAA and IAA was suggested for *in vitro* multiplication of *Arctostaphylos uva-ursi* (Kada *et al*., 1991) and *Mammillaria san-angelensis* (Rubluo *et al*., 2002).

Based on shoot formation rate and also on number of concentrations that induced more shoots than hormone free medium, NAA was not suitable hormone during the first subculture. However, during the second and third subculture, 8 of the 10 NAA concentrations produced more shoots than hormone free and at 3 concentrations, NAA was even better than BAP and IAA. Comparing the different hormone types each at equal concentration showed that BAP was better than both of NAA and IAA only at one level

(2.5 mg/l), less than both at two levels (4.0 and 5.0 mg/l) and equal to both at two levels (1.5 and 2.0 mg/l). However, limiting the comparison to only two hormone types at one time showed that the BAP was better than NAA at four (2.5, 3.0, 3.5 and 4.5 mg/l), less at other four levels (0.5, 1.0, 4.0 and 5.0 mg/) and equal at two levels (1.5 and 2.0 mg/l) and was better than IAA at two (0.5 and 2.5 mg/l), less at other two levels (4.0 and 5.0 mg/l) and equal at six levels (1.0, 1.5, 2.0, 3.0, 3.5 and 4.5 mg/l). Hence, if a range of 1 to 4 different concentrations were arbitrarily picked, either one of these three hormones could be recommended as the best depending on the tested concentrations. That is for valid test a minimum of at least 5 different concentrations should be used to investigate the effect of different hormones. Omokoio *et al.* (2001) and Fitchet (1990a) reported that at equal concentration of both hormones KN resulted in higher shoot formation rate than BAP while Mathew and Rangan (1981, 1979) reported that KN resulted in higher rate than hormone free medium but was less than BAP. On the contrary, our results (Table, 3.15) indicated that KN is not a suitable hormone for *in vitro* proliferation of Moris pineapple. Seven out of ten KN concentrations resulted not only in lower shoot formation than BAP but also were not significantly different from hormone free medium.

The most usual practice during multiplication is using the same hormone treatment for several subcultures and reporting the rate of shoot formation as average overall subcultures. Following this approach not only would not lead to optimization of shoot formation rate and total but also would result in overestimation of total shoot production.

Using of rate as the sole parameter of assessment could not distinguish between treatments of different total shoot production. However, assessment based on total shoot production distinguished between treatments of equal rate and specified the best treatment to one hormone type and a single concentration. For instance, BAP at 2.0, 3.0 and 3.5, NAA at 0.5, 2.0 and 5.0 and IAA at 2.0, 3.0 and 4.0 mg/l. each resulted in equal rate of 6 shoots per explant but significantly different total of shoots. It is worth noting that estimation of total shoot production by multiplication of average shoot formation at each subcultures indicated production of 160, 210, 180, 88, 160 135, 72, 200, 104 and 60 shoots after 3 subcultures in medium enriched with BAP at 2.0, 3.0 and 3.5, NAA at 0.5, 2.0 and 5.0 and IAA at 2.0, 3.0 and 4.0 mg/l respectively while using the shoot formation average overall subcultures (6^{3}) for estimation of total shoots all treatments are expected to produce equal total of 216 shoots (Table, 3.15). The difference between the two methods of estimation ranged from a negligible difference of just 6 shoots (216 -210) in medium enriched with BAP at 3.0 mg/l to a huge difference of 156 shoots (216- 60) in medium enriched with IAA at 4.0 mg/l. This clearly indicates that reporting of only the average of shoot formation overall subcultures as it is usually done by all researches may lead to an overestimation of the total shoot production and serious legal problem concerning the delivery time of propagules. Total shoots was used for evaluation of pineapple cultures (Firoozabady and Gutterson, 2003; Almedia, *et al*., 2002; Devi, *et al*., 1997) but at one fixed hormone treatment.

Generally, at each subculture, there were different optimal of hormone types and concentration (Table, 3.15). Of all treatments, the highest rate (7 shoots) during the first subculture obtained in medium with BAP at 3.0 mg/l. At the second subculture the highest rate (13 shoots) obtained in medium with IAA at 2.0 and 3.5 mg/l. At the third subculture explants treated with NAA at 2.5 and 3.0 mg/l gained the lead with 7 shoots per explant. If the effect of different types of hormones over subcultures remained stable when applied in alternative sequence, BAP at 3.0 mg/l during the first, IAA at 2.0 mg/l during the second and NAA at 2.5 mg/l during the third subculture would be the best sequence of hormone for the highest proliferation of Moris pineapple. Application of different hormone types or concentrations at different subculture is not usual. However, Nielsen, *et al*. (1995) recommended sequenced use of BAP and TDZ as a mean of overcoming the decline of shoot formation of *Miscanthus x ogiformis* over subcultures.

The lowest cost per shoot (22 cents) obtained in medium enriched with NAA at 2.5 mg/l (Table, 3.16). However, the total shoots were 35 shoots only. Medium enriched with BAP at 3.0 mg/l increased the cost per shoot to 25 cents but produced the highest total of shoots (210 shoots) and resulted in the highest total cost (RM 72.23). The total cost of medium enriched with IAA at 0.5 mg/l was equal to the total cost of the medium enriched with NAA at 2.5 (RM 9.88) but produced only 5 shoots and resulted in the highest cost per shoot (153 cents). Partitioning the total cost to different cost items (Table, 3.16, Figure, 3.5) showed that the highest percentage of cost (40 %) was mainly due to maintaining the

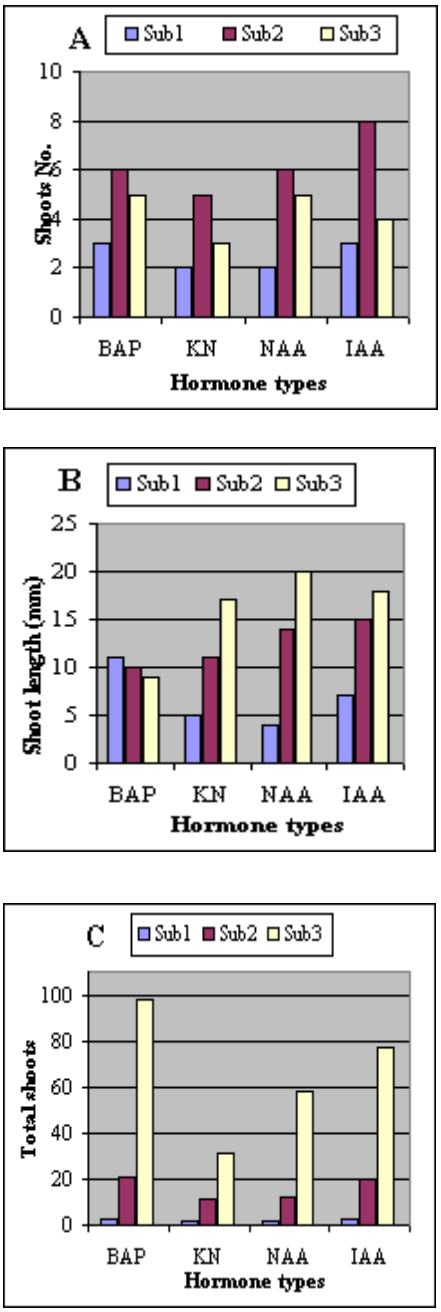


Figure (3.4). Effect of hormone types and subcultures on shoot formation (A), shoot length (B) and total shoot production of Moris pineapple cultured on agar solidified full strength MS medium enriched with sucrose at 30 g/l and singly applied BAP, KN, NAA and IAA each at10 different concentrations and incubated for 60 days

Table 3.15 Effect of hormone types, concentrations and subcultures on the *in vitro* shoot formation, shoot length, total shoots, total cost and cost per shoot of Moris pineapple

Hormones		**Shoot formation per explant**				**Shoot length**	**Total shoots**		**Cost (RM)**	
Types	mg/l	Sub1	Sub2	Sub3	Average	(mm)	A	B	Total	/shoot
BAP	0	3 efg	3efg	3 efg	3 BC	21 a	27 hij	27	18.03	0.51
	0.5	3 efg	8 bcde	4 defg	5 AB	13 abcdefg	96 def	125	40.63	0.30
	1	3 efg	5 cdefg	4 defg	4 ABC	14 abcdefg	60 efghij	64	27.07	0.33
	1.5	4 defg	6 cdefg	5 cdefg	5 AB	11 bcdefgh	120 cde	125	42.25	0.25
	2	5 cdefg	8 bcde	4 defg	6A	9 cdefgh	160 abc	216	66.98	0.30
	2.5	3 efg	8 bcde	5 cdefg	5 AB	12 abcdefgh	120 cde	125	40.63	0.24
	3	7 bcdef	6 cdefg	5 cdefg	6A	8 defgh	210 a	216	72.23	0.25
	3.5	4 defg	9 bcd	5 cdefg	6A	9 cdefgh	180 ab	216	73.39	0.29
	4	1 g	2 fg	6 cdefg	3 BC	10 bcdefgh	12 ij	27	5.75	0.38
	4.5	1 g	6 cdefg	5 cdefg	4 ABC	6 gh	30 ghij	64	11.39	0.29
	5	2 fg	2 fg	4 defg	3 BC	7 efgh	16 ij	27	9.38	0.48
KN	0.5	3 efg	10 abcd	3 efg	5 AB	7 efgh	90 defg	125	49.67	0.39
	1	1 g	1 g	3 efg	2 C	7 fgh	3 j	8	4.74	1.18
	1.5	3 efg	8 bcde	2 fg	4 ABC	15 abcdefg	48 fghij	64	40.63	0.61
	2	2 fg	4 defg	3 efg	3 BC	11 bcdefgh	24 hij	27	15.41	0.49
	2.5	2 fg	3 efg	4 defg	3 BC	13 abcdefg	24 hij	27	12.39	0.41
	3	1 g	3 efg	4 defg	3 BC	11 bcdefgh	12 ij	27	6.87	0.47
	3.5	2 fg	4 defg	3 efg	3 BC	14 abcdefg	24 hij	27	15.41	0.49
	4	2 fg	7 bcdef	3 efg	4 ABC	9 cdefgh	42 fghij	64	24.45	0.43
	4.5	1 g	4 defg	4 defg	3 BC	10 bcdefgh	12 ij	27	8.37	0.56
	5	2 fg	4 defg	4 defg	3 BC	10 bcdefgh	32 ghij	27	15.41	0.37

Means of the same parameter followed by same letter were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range test

Each treatment consisted of 9 explants cultured individually on 20 ml of agar solidified full strength MS enriched with sucrose at 30 g/l and incubated for 60 days.

A. total estimated by multiplication of shoot formation rate at each subculture (sub1x sub2 x sub3)

B.Total estimation by using the shoot formation rate overall subcultures $(6)^3$

RM Malaysian Ringgit (1 USA $ = RM 3.25).

Total cost included only the variable items of cost (Jars, MS, agar, sucrose, hormones, electricity cost of autoclave, laminar operation and incubation room and the wages of labor). Cost of jars was not included in the estimated cost per shoot.

Table 3.15 (continued).

Hormones		**Shoot formation per explant**				**Shoot length**	**Total shoots**		**Cost (RM)**	
Types	mg/l	Sub1	Sub2	Sub3	Average	(mm)	A	B	Total	/shoot
NAA	0.5	2 fg	11 abc	4 defg	6A	17 abcd	88 defghi	216	36.50	0.29
	1	1 g	7 bcdef	6 cdefg	5 AB	12 abcdefgh	42 fghij	125	12.89	0.23
	1.5	1 g	7 bcdef	5 cdefg	5 AB	7 fgh	35 ghij	125	14.40	0.27
	2	4 defg	7 bcdef	5 cdefg	6A	12 abcdefgh	160 abc	216	53.81	0.24
	2.5	1 g	5 cdefg	7 bcdef	4 ABC	7 fgh	35 ghij	64	9.88	0.22
	3	1 g	4 defg	7 bcdef	4 ABC	12 abcdefgh	28 hij	64	8.37	0.24
	3.5	1 g	4 defg	4 defg	3 BC	18 abc	16 ij	27	8.37	0.42
	4	1 g	7 bcdef	6 cdefg	5 AB	16 abcdef	42 fghij	125	12.89	0.23
	4.5	2 fg	5 cdefg	3 efg	3 BC	12 abcdefgh	30 ghij	27	18.42	0.46
	5	3 efg	9 bcd	5 cdefg	6A	17 abcde	135 bcd	216	45.15	0.24
IAA	0.5	1 g	5 cdefg	1 g	2 C	4 h	5 j	8	9.88	1.53
	1	3 efg	3 efg	5 cdefg	4 ABC	19 ab	45 fghij	46	18.03	0.31
	1.5	3 efg	7 bcdef	5 cdefg	5 AB	11 bcdefgh	105 cdef	125	36.11	0.25
	2	2 fg	12 ab	3 efg	6A	15 abcdefg	72 efghi	216	39.52	0.39
	2.5	2 fg	7 bcdef	3 efg	4 ABC	14 abcdefg	42 fghij	64	24.45	0.42
	3	4 defg	10 abcd	5 cdefg	6A	16 abcdef	200 a	216	65.86	0.23
	3.5	2 fg	13 a	4 defg	6A	13 abcdefgh	104 cdef	216	42.53	0.29
	4	1 g	12 ab	5 cdefg	6A	11 bcdefgh	60 efghij	216	20.43	0.25
	4.5	6 cdefg	4 defg	3 efg	4 ABC	15 abcdefg	72 defghi	64	43.99	0.45
	5	5 cdefg	8 bcde	3 efg	5 AB	14 abcdefg	120 cde	125	66.98	0.39

Means of the same parameter followed by same letter were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range test

Each treatment consisted of 9 explants cultured individually on 20 ml of agar solidified full strength MS enriched with sucrose at 30 g/l and incubated for 60 days.

A. total estimated by multiplication of shoot formation rate at each subculture (sub1x sub2 x sub3)

B.Total estimation by using the shoot formation rate overall subcultures $(6)^3$

RM Malaysian Ringgit (1 USA $ = RM 3.25).

Total cost included only the variable items of cost (Jars, MS, agar, sucrose, hormones, electricity cost of autoclave, laminar operation and incubation room and the wages of labor). Cost of jars was not included in the estimated cost per shoot.

Table (3.16) .Multiplication cost items of Moris pineapple after three cycles of 60 days long multiplication

Cost items		**Hormones (mg/l)**					
		BAP	IAA	NAA	Free	IAA	KN
		3	3	2.5	0	0.5	1
		Cost (RM)					
Jars	Jars	21.00	20.00	2.50	4.50	3.00	1.50
Medium	MS	8.00	7.20	1.12	2.08	1.12	0.48
	Agar	3.86	3.48	0.54	1.00	0.54	0.23
	Sucrose	2.64	2.38	0.37	0.69	0.37	0.16
	Hormone	0.24	0.04	0.00	0.00	0.00	0.01
Electricity	Incubation	20.00	18.00	2.80	5.20	2.80	1.20
	Autoclave	3.75	3.38	0.53	0.98	0.53	0.23
	Laminar	2.36	2.15	0.57	0.82	0.29	0.40
Labor	Jclean	0.89	0.56	0.33	0.44	0.33	0.22
	Mprep	1.67	1.50	0.23	0.43	0.23	0.10
	Culturing	8.33	7.50	1.17	2.17	1.17	0.50
	Total (+ jars)	72.74	66.17	10.16	18.31	10.16	5.03
	Total (- jars)	51.74	46.17	7.66	13.81	7.66	3.53
	Shoots	210	200	35	27	5	3
	Cost per shoot (+ jar)	0.35	0.33	0.29	0.68	2.03	1.68
	Cost per shoot (- jar)	0.25	0.23	0.22	0.51	1.53	1.18

RM Malaysian Ringgit (1 USA $ = RM 3.25).
Total cost included only the variable items of cost (Jars, MS, agar, sucrose, hormones, electricity cost of autoclave, laminar operation and incubation room and the wages of labor).
The medium was agar solidified (7 g/l) full strength MS medium enriched with sucrose at 30 g/l adjusted to pH 5.7 and dispensed at 20 ml per glass jars and autoclaved at 121 ^{0}C and 1.5 kg/ cm^{2} for 25 minutes.
Culture incubated for 60 days at constant temperatire 25 ^{0} C and 16 hours of light provided by cool white florescence lamps.
Jclean = (jars cleaning), Mprep. = (medium preparation)
(+ jar) Jar cost included
(- jar). Jar cost not included
Culturing (separation and culturing of shoots under laminar)
Incubation, Autoclave, laminar (electricity cost of operation of incubation room, autoclave and laminar)

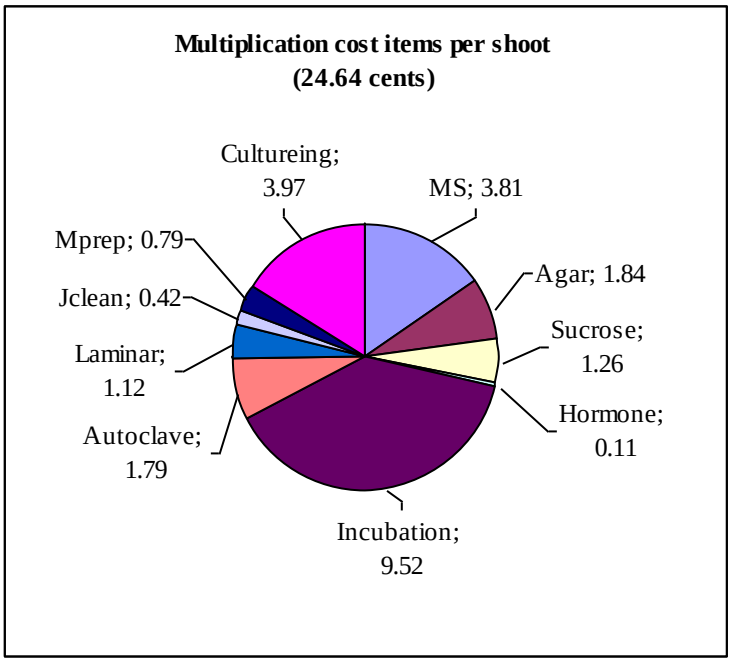


Figure (3.5). Multiplication cost items per shoot of Moris pineapple after 3 subcultures on 20 ml of agar solidified (7 g/l) full strength MS medium enriched with sucrose at 30 g/l and BAP at 3.0 mg/l and incubated under constant temperature 25 ^{0}C and 16 hours of light for 60 days.
Glass jar cost was not included (reusable item) and cost are in Malaysian cents
Jclean = (jars cleaning), Mprep. = (medium preparation).
Culturing (separation and culturing of shoots)
Laminar, Autoclave and Incubation (electricity cost for operation of laminar, autoclave and incubation room)

incubation room at a constant 16 hour of light and temperature at 25 ^{0}C. Labor wages (culturing) and MS medium each caused 16 and 15 % of total cost and cost per shoot. Generally, of each one Ringgit, 52 cents were spent for electricity (autoclave, laminar and incubation), 27 cents for medium (MS, agar and sucrose) and 21 cents for labor (culturing, preparing media and cleaning jars). The cost of these three items could be reduced by many different approaches and a cost per shoot lower than 22 cents is definitely obtainable. For instance, in tropical areas the electricity cost of incubation room could be completely eliminated simply by adoption and improving of the outdoor incubation procedure suggested by Be and Debergh (2006). The cost could also be reduced further by using liquid instead of agar solidified medium and optimization of multiplication factors as medium strength, sucrose content, explant density and medium volume per culture. Once a system of highest multiplication and minimum cost per shoot develop, the cost reduction approaches applied also to the rooting and acclimatization for large scale production of propagules at lowest cost.

3.2.8. Effect of sucrose, pH and medium states on Moris pineapple proliferation and growth

Analysis of variance shows that the shoot formation and the shoot length were under direct effect of the medium states ($p \leq 0.0166$ and $p \leq 0.0001$) and sucrose concentrations ($p \leq 0.0002$ and ≤ 0.00003). However, while the sucrose effect on shoot formation was influenced by significant interaction with pH ($p \leq 0.0039$ and ≤ 0.00001), the medium states effect on shoot formation was independent of pH ($p \leq 0.3936$) and sucrose ($p \leq$

0.0764). The medium state effect on shoot length was influenced by the medium sucrose content ($p \leq 0.0141$) but independent of pH ($p \leq 0.0764$). Medium pH had no direct independent effect on both shoot formation (p 0.3031) and shoot length ($p \leq 0.7794$) but interacted significantly with medium sucrose content ($p \leq 0.0039$ and $p \leq 0.00001$). On the other hand, both the shoot formation ($p \leq 0.1520$) and shoot length (≤ 0.2460) were not affected by the collective interaction of the three factors. Overall, liquid medium resulted in formation of more (4.7 shoots) and longer (41 mm) shoots (Figure, 3.6A and D) than semi solid and solid (4 shoots each 30 mm long). Enrichment of the medium with sucrose at 30 g/l resulted in the highest (4.83 shoots) and at 10 g/l in the lowest shoot formation (3.5 shoots). Sucrose at 20 and 40 g/l resulted in intermediate rate of 4.58 and 4.0 shoots per explant respectively (Figure, 3.6B). The longest shoots (43 mm), on the other hand, obtained in medium enriched with sucrose at 20 g/l and the shortest (22 mm) in medium enriched with sucrose at 40 g/l. Sucrose at 10 and 30 g/l resulted in intermediate shoot length of 32 and 38 mm respectively (Figure, 3.6E). The medium pH had no significant direct effect on the process of shoot formation ($p \leq 0.3031$) and shoot elongation ($p \leq 0.7994$). Overall, the different pH adjustments resulted in formation of 4 shoots each 34 mm long (Figure, 3.6C and F). However, the pH influenced both of shoot formation ($p \leq 0.0039$) and shoot length ($p \leq 0.00001$) via interaction with sucrose content of the medium (Table, 3.17).

Liquid medium enriched with sucrose at 20, 30 and 40 g/l (Figure, 3.7A) resulted in more shoots (5.8, 5.3 and 4.3 shoots) than solid medium (4.0, 5.0 and 3.5 shoots). On the contrary, in medium enriched with sucrose at 10 g/l, solid medium resulted in formation of more shoots (3.8 shoots) than liquid (3.5 shoots). Liquid medium enriched with sucrose at 10, 20 and 30 g/l resulted in longer shoots (46, 53 and 45 mm respectively) than solid medium (23, 32 and 32 mm) while in medium enriched with sucrose at 40 g/l (Figure,

3.7B), solid medium resulted in longer shoots (27 mm) than liquid medium (20 mm). Overall sucrose, medium states had no effect on shoot formation. Equal shoot formation (4 shoots) obtained in solid, liquid and semi solid media irrespective of pH adjustments (Figure, 3.7C). However, while solid and liquid media adjusted to pH 5.0 and 6.5 produced shoots of equal length (35 mm), liquid medium adjusted to pH 5.7 and 6.0 resulted in longer shoots (35 and 47 mm) than that of solid (28 and 28 mm) medium (Figure, 3.7D). Similar, overall medium states, the highest shoot formation in medium enriched with sucrose at 20 and 30 g/l (6.0 and 5.7 shoots) obtained in medium adjusted to pH 5.0 while in medium enriched with sucrose at 10 and 40 g/l, the highest shoot formation (3.9 and 5.0 shoots) obtained in medium adjusted to pH 6.5 and 5.7 respectively (Figure, 3.7E). In medium enriched with sucrose at 20, 30 and 40 g/l, the shoot formation decreased as the pH increased while in medium enriched with sucrose at 10 g/l, the shoot formation increased as the pH increased. The longest shoots in medium enriched with 10 and 30 g/l (38 and 47 mm) obtained in medium adjusted to pH 5.0 while in medium enriched with sucrose at 20 and 40 g/l, the longest shoots (50 and 38 mm) obtained in medium adjusted to pH 6.0 and 6.5 respectively (Figure, 3.7F). In medium enriched with sucrose at 10 and 30 g/l, the shoot length decreased as the pH increased while in medium enriched with sucrose at 40 g/l, the shoot length increased as the medium pH increased. The lowest shoot formation (3 shoots) obtained in medium enriched with sucrose at 10 g/l and adjusted to pH 5.0 and the shortest shoots (12 mm) obtained in medium enriched with sucrose at 40 g/l and adjusted to pH 5.7. The highest shoot formation (7 shoots) and elongation (50 mm long shoots), on the other hand, obtained in medium enriched with sucrose at 20 g/l and adjusted to pH 5.0 and 6.0 respectively.

The highest shoot formation of all combinations (7 shoots) obtained in liquid medium enriched with sucrose at 20 g/l and solid medium enriched with sucrose at 30 g/l and both

adjusted to pH 5.0 (optimal treatment). The lowest shoot formation of 3 shoots per explant obtained in solid, semi solid and liquid media of different sucrose content and pH. Equal formation of 7, 6, 5, 4 and 3 shoots obtained in liquid and solid medium, but at different combinations of sucrose and pH (Table, 3.18). At fixed sucrose concentration of 10 g/l, adjustment of the medium to different pH (pH 5.0, 6.0 and 6.5) resulted in equal shoot formation (4 shoots) in both solid and liquid medium while when the media were adjusted to pH 5.7, the solid medium resulted in more shoots (4 shoots) than liquid medium (3 shoots). Fixing the sucrose at 40 g/l and adjusting the media to pH 5.7, solid medium produced also more shoots (5 shoots) than liquid medium (4 shoots) while adjusting to pH 6.0 and 6.5, liquid medium produced more shoots (4 and 6 shoots) than solid (3 and 3 shoots). Enrichment the media with sucrose at 20 and 30 g/l, and adjusting pH to 5.7, 6.0 and 6.5, liquid medium produced more shoots than solid medium while in media adjusted to pH 5.0, solid medium on the contrary produced more shoots than liquid medium. Generally at equal concentration of sucrose, adjusting the medium to higher pH decreased the shoot formation in solid medium but increased the shoot formation in liquid medium.

Similar, fixing the medium pH at 5.0, equal shoot formation (3 shoots) obtained in both solid and liquid medium enriched with sucrose at 10 and 40 g/l while in medium enriched with sucrose at 20 and 30 g/l, solid produced more shoots than liquid. At fixed pH of 5.7, solid medium enriched with sucrose at 10 and 40 g/l produced more shoots (4 and 5 shoots) than liquid (3 and 4 shoots) while in medium enriched with sucrose at 20 and 30 g/l, liquid medium produced more (6 and 5 shoots) shoots than solid medium (3 and 4 shoots). At fixed pH of 6.0, equal shoot formation (4 shoots) obtained in solid and liquid media enriched with sucrose at 10 and 20 g/l (low sucrose enrichment) while in media enriched with sucrose at 30 and 40 g/l (high enrichment) liquid produced more (6 and 4 shoots) shoots than solid medium (5 and 3 shoots). At pH 6.5, liquid enriched with sucrose

at 20, 30 and 40 g/l produced more (6, 5 and 6 shoots) shoots than solid medium (3, 4 and 3 shoots) and in media enriched with sucrose at 10 g/l equal shoot formation (4 shoots) obtained in both medium states. Increasing the pH from 5.0 to 6.0 increased the shoot formation of liquid medium enriched with sucrose at 30 g/l from 5 to 6 shoots but decreased the shoot formation of the solid medium enriched with sucrose at 20 from 6 to 3 shoots and in medium enriched with sucrose at 30 g/l from 7 to 5 shoots (Table, 3.18). Adjusting the solid medium to pH higher than 5.0, decreased the shoot formation irrespective of the medium sucrose content. On contrary, in liquid medium, adjusting the pH to 6.0 improved the shoot formation in medium enriched with sucrose at 30 g/l and 40 g/l from 5 to 6 shoots and from 3 to 6 shoots respectively, but decreased the shoot formation in medium enriched with sucrose at 20 g/l from 7 to 4 shoots.

The best combinations for longest shoots (61 mm) were liquid medium enriched with sucrose at 10 and 20 g/l and both adjusted to pH 6.0 (optimal treatment for shoot length) while the shortest shoots (5 mm) obtained in semi solid medium enriched with sucrose at 40 g/l and adjusted to pH 6.5 (Table, 3.18). At fixed sucrose enrichment of 20 g/l and all pH adjustments and at fixed sucrose of 10 and 30 g/l, and all pH adjustments except 6.5, liquid media resulted in longest shoot, solid media in shortest and intermediate shoot length obtained in semi solid medium. On the contrary, at sucrose 40 g/l, at all pH except 6.5, solid medium resulted in the longest while semi solid in the shortest shoots and intermediate shoot length obtained in liquid medium. In media enriched with sucrose at 10 g/l and adjusted to pH 6.5, solid medium resulted in the longest (34 mm) and semi solid in the shortest (17 mm) while intermediate shoots (28 mm) obtained in liquid medium. However, when the sucrose enrichment was 20 g/l, the longest shoots (58 mm) obtained in liquid medium and the shortest shoots (29 mm) in solid while semi solid medium resulted in an intermediate (42 mm) shoot length. In media enriched with sucrose at 30 g/l equal

shoot length (27 mm) obtained in solid and liquid medium and shortest shoots (17 mm) obtained in semi solid medium while in media enriched with sucrose at 40 g/l, semi solid medium resulted in the longest shoots (48 mm) while solid and liquid resulted in the shortest (38 mm) shoot length.

At fixed sucrose concentration of 10 and 20 g/l, increasing the pH from 5.0 to 6.0 increased the shoot length in liquid medium from 45 to 60 mm but decrease the shoot length in solid medium from 23 to 20 and from 36 to 33 mm. The shoot length of semi solid medium enriched with sucrose at 10 g/l decreased from 42 to 28 mm and on medium enriched with sucrose at 20 g/l increased from 43 to 55 mm. The shoot length in medium enriched with sucrose 30 g/l increased while that of medium enriched with sucrose 40 g/l decreased as the medium pH increased. At fixed pH of 5.0, the shoot length in both of solid and liquid medium respectively increased from 23 and 45 mm to 41 and 56 mm as the sucrose increased from 10 to 30 g/l while in semi solid the shoot length remained about the same (42 to 46 mm). On all of the three states, solid, liquid and semi solid, increasing the sucrose concentration from 30 to 40 g/l caused a sharp decline in shoot length from 41, 46 and 56 mm to 22, 12 and 7 mm respectively. Similar, at fixed pH of 5.7, the shoot length in solid and semi solid media increased from 18 and 22 mm to 46 and 45 mm while in liquid medium the shoot length remained about the same (51 mm). In media enriched with sucrose at 40 g/l, the shoot length declined to 14, 14 and 12 ml respectively. At fixed pH of 6.0, the shoot length in liquid and semi solid media increased from 20 and 28 mm to 33 and 55 mm as the sucrose increased from 10 to 20 g/l and decreased afterwards. On contrary, at fixed pH 6.5, the shoot length in solid medium decreased from 34 to 26 mm as the sucrose increased from 10 to 30 g/l and increased to 38 mm in medium enriched with sucrose at 40 g/l. In semi solid and liquid media enrichment with sucrose at 10 and 30 g/l resulted in shorter shoots than enrichment with sucrose at 20 and 40 g/l.

Estimation of cost per shoot (Table, 3.19) indicated that the lowest cost per shoot (RM 12) could be obtained using solid MS enriched with sucrose at 30 g/l and liquid medium enriched with sucrose at 20 g/l, both adjusted to pH 5.0. Dividing the cost into the different cost items (Figure, 3.8) indicated that jars, labor salary, MS medium and electricity of incubation room were the major cause of high cost per shoot. Jars are reusable item and may not include in the estimation of cost per shoots. However, the autoclave can only hold three baskets full of 100 jars at one time (autoclaving cycle) and hence using of jars would increase the autoclaving cost.Alternatively, using of 2 litter glass bottles for medium autoclaving and dispensing the medium under laminar into culture tubes would allow autoclaving of 6 liters of medium per autoclave cycle and reduced the cost of autoclave operation but it would increased the laminar operation time for dispensing of medium into the culture tubes.

Whether liquid or solid medium is better for proliferation of Moris depended on the sucrose content and pH of the medium and the parameter of assessment. The optimum medium states varied at different sucrose concentrations and the optimum sucrose varied at different pH adjustment and vice versa. Sucrose at 10 and 40 g/l were not appropriate for Moris proliferation and selection of proper pH is very essential. Adjusting the medium to different pH at equal concentration of sucrose and using of different sucrose concentrations at fixed pH could double or cause drastic loss in shoot formation and growth capacity. Different medium states had different sucrose concentration for optimal shoot formation and shoot length. On the same medium state the highest shoot formation and longest length obtained in equal sucrose concentration but at different pH adjustments and on different states the highest shoot formation and longest length obtained in different sucrose concentrations but equal pH. The best combination for the highest shoot formation (7 shoots) in solid and liquid were sucrose at 30 and 20 g/l respectively and pH 5.0. Keeping

the same concentration of sucrose but readjusting the pH to 5.7 and 6.0 resulted in the longest shoot in solid (46 mm) and liquid (61 mm) media respectively. Liquid state was in generally super than solid and sucrose at 40 g/l inhibited the shoot formation. However, the superiority of liquid state and the inhibition of high sucrose content could be blocked and even reversed by pH adjustment.

In almost all of the reported comparisons of solid and liquid medium effect on pineapple *in vitro* cultures, the sucrose and agar were kept fixed at 30 and 6 to 8 g/l respectively and the pH at 5.7 in both media. Under these conditions, using of liquid medium resulted in more shoots per explant (Be and Debergh, 2006; Aydieh *et al*., 2000; Fitchet, 1990a; Mathew and Rangan, 1979; Dewald *et al*., 1988; Fernando, 1986) and higher total (Firoozabady and Gutterson; 2003; Almeida *et al*., 2002) than solid medium. The results of this study confirmed their findings but revealed that the superiority of liquid medium depended on the sucrose content and pH of the medium. Equal proliferation obtained in solid and in liquid medium at fixed and at different combination of sucrose and pH and liquid was better than solid at some and solid was better than liquid medium at other combinations (Table, 3.18). Out of the 16 combinations of sucrose and pH, the liquid medium resulted in more shoots than solid and semi solid in 8 combinations (50 % of the cases). However, at other 5 combinations (31 %), the shoot formation in liquid was even less than that of solid. At 5 combinations (31 %) the shoot formation in liquid medium was equal and at 2 combinations (13 %) was less than that in semi solid medium. It is important to note that the shoot formation obtained in liquid medium enriched with sucrose at 30 g/l and pH 5.7 could even be higher if the medium was adjusted to pH 6.0 instead of pH 5.7 or by keeping the pH at 5.7 but decreasing the sucrose to 20 g/l instead of 30 g/l and by increasing both, the sucrose to 40 g/l and pH to 6.5.

The shoot formation in both solid and liquid medium ranged from minimum of 3 to maximum of 7 shoots and each medium state had different optimal sucrose concentrations and pH adjustments. Yet it appeared that shoot formation in solid medium had a very critical requirement and narrow range while the liquid medium had a wider range of sucrose-pH combinations. In solid and semi solid medium 75 % of the sucrose and pH combinations (12 of 16) resulted in less than 5 shoots and 25 % and 13 % in more than 5 shoots respectively while in liquid medium 50 % of the combinations resulted in less than 5 and 50 % in more than 5 shoots. Selection of proper sucrose and pH is critical and very specific in solid than in liquid medium. Furthermore, not only the different states had different optimal sucrose and pH but the same state had different optimal for different growth parameter. The highest proliferation and shoot elongation in solid medium obtained in medium enriched with sucrose at 30 g/l but of different pH. Highest shoot formation in medium adjusted to pH 5.0 and longest shoots in medium adjusted to 5.7. In semi solid the highest rate obtained in medium enriched with sucrose at 40 g/l and adjusted to pH 5.7 but the longest shoot in medium enriched with sucrose at 20 g/l and adjusted to pH 6.0. The highest proliferation and shoot elongation in the liquid medium, on the other hand, obtained in medium enriched with sucrose at 20 g/l but the highest shoot formation in medium adjusted to pH 5.0 and longest shoots in medium adjusted to pH 6.0. Hence, adopting of one single sucrose-pH treatment for both states and different growth parameters is certainly not optimal for both, particularly solid medium. In fact, it could drastically reduce the proliferation in one medium state while doubling the proliferation in other one. According to the results of our study fixing the pH at 5.7 (the most common pH adjustment) is not proper practices for Moris pineapple. It reduced the possibly obtainable rate of shoot formation in medium enriched with sucrose at 20 and 30 g/l by about 50 %. Arbitrary using of a fixed sucrose concentration and pH would not allow estimation and

exploitation of the inherited proliferation capacity of the explants. Since, the optimal pH for elongation is different from that for shoot formation, it is advisable that at the last multiplication cycle just before transferring the shoots to rooting and acclimatization to change the pH of liquid to 6.0 and that of solid to 5.7 to enhance the shoot elongation process.

Almost, in all of the cases in which solidified medium was recommended for pineapple, agar at 6 and 8 g/l was used solidification agent tested. Other agents such as phytagel (Ko *et al*., 2006; Smith *et al*.2002) and gelrite (Firoozabady and Moy, 2004; Firoozabady and Gutterson, 2003) were rarely used. Sripaoraya *et al*. (2003) recommended agar for axillary bud explants, but for leaf explants and harvested cells from suspension culture, agarose at 4 g/l were favored over agar. No comparisons between agar concentrations or different gelling agents were reported for pineapple. Our study indicated thatat lower sucrose concentration (10 to 30 g/l) higher agar concentration (7 g/l) was better than lower concentration (3.5 g/l) at 5 combinations (31 %) and equal at 6 (50 %) and less at 1 (9 %) of the combinations of sucrose and pH. At higher sucrose (40 g/l), lower agar resulted in higher shoot formation than higher agar concentration in medium adjusted to low pH (5.0 and 5.7) and equal shoot formation at higher pH (6.0 and 6.5). Since agar is more expensive than sucrose, lower agar concentration (semi solid state) may favor over solid in spite of the high sucrose concentrations.

Sucrose is an indispensable component of medium. *In vitro* culturing of plant material could not be done without supplement of carbon source. Sucrose at 30 g/l and pH at 5.7 was the most recommended combination for pineapple *in vitro* culture. Soneji *et al*. (2002a) reduced the sucrose to 20 g/l but kept the pH at 5.7. Fitchet (1990a) and Teixeira *et al*. (2006) kept the sucrose at 30 g/l but the first lower the pH to 5.0 while the second increased the pH to 6.5. Kofi and Adachi (1993) and (Almeida *et al*., 1997) kept the pH at

5.7 but increased the sucrose to 35 and 40 g/l respectively. Surprisingly in all of these studies, the effect of different concentrations of sucrose was neither compared at same or different medium states nor at different pH adjustments. Although, a reasonable rate was reported, the sucrose and pH combination could not be assumed as the optimal one. The results of our study demonstrated that the effect of an ignored factor such as pH of the medium could substantially reduce the amount of sucrose and increased the rate of multiplication of pineapple. Compared to the common practice of enrichment the medium with sucrose at 30 g/l and adjusting the pH to 5.7, maintaining the same sucrose concentration, but decreasing the pH to 5.0 and lowering both, (sucrose to 20 g/l and pH to 5.0) increased the shoots formation of solid medium by 100 %. Maintaining the same sucrose concentration and increasing the pH to 6.0 and lowering both, (sucrose to 20 g/l and pH to 5.0) improved the shoot formation in liquid medium by 17 to 20 %. That is in both medium states the cost of medium (sucrose) could be reduced by 30 % and the cost per shoot in solid by 34 % and in liquid medium by 27 %. Besides increasing shoot formation and reducing the medium cost and the cost per production unit (shoot), using of lower concentration of sucrose would most likely improve photoautotrophy and increase out door plantlet survival.

Medium states, sucrose and pH played important role in the process of shoot formation and elongation. However, that role is not clearly understood yet. It is generally assumed that the promotion effect of liquid medium is due to accessibility of medium component to the explants. The result (Table, 3.18) showed that the superiority of liquid state (promotion effect) could be blocked and even reversed by pH and sucrose content. Liquid medium enriched with sucrose at 20 g/l at all pH except 6.0 and with sucrose at 30 g/l at all pH except pH 5.0 resulted in more shoots than solid medium. Adjusting the pH to 6.0 in the medium enriched with sucrose at 20 g/l blocked the promotion effect of liquid medium

(equal shoots obtained in both medium states) while adjusting the pH 5.0 in the medium enriched with 30 g/l reversed the situation and solid promoted more shoots than liquid. That is the pH and sucrose directly or indirectly affected the liquid state promotion of shoot formation. If the accessibility was the cause of difference between solid and liquid media, the statistical analysis should have shown significant interaction. Neither the interaction of medium states with pH and sucrose each alone nor the collective interaction of the three factors together was significant. The effect of the medium states was direct and independent of the sucrose concentrations and pH adjustments.

Compared to sucrose at 10 g/l and pH 5.0, sucrose at 20 g/l doubled the rate of shoot formation in both medium states (Table, 3.18). Adjusting the medium pH to 6.0, blocked the sucrose promotion effect in both states (less shoots and equal in both states) while adjusting to other pH (5.7 and 6.5) blocked the sucrose effect in solid (less shoots than that at pH 5.0) but did not effect the shoot formation in liquid medium (equal to that at pH 5.0). In medium adjusted to pH 5.0, sucrose at 40 g/l inhibited the shoot formation and blocked the promotion effect of the liquid medium state. The shoot formation declined to the lowest rate and equal shoots obtained in both states (3 shoots). However, while adjusting the liquid medium pH to 6.5 overcome the high sucrose inhibition of shoot formation (rate increased to 6 shoots) it failed to overcome the high sucrose inhibition of the shoot formation in solid medium (rate remained 3 shoots). Since neither of the sucrose and pH interacted with medium state, the effect of both must be through affecting the explants metabolic and physiologic activity (enzymes activation). The medium states effect, on the other hand, is most likely through providing some sort of physical and chemical environment for the activation of those enzymes.The results of this study could help in selection of specific combinations of sucrose concentrations and pH adjustments for identification, isolation and kinetic study of the enzymes involved in shoot formation of

Table (3.17). Main and interaction effect of medium states, sucrose concentrations and pH adjustments on the *in vitro* shoot formation and shoot length of Moris pineapple

Factors	df	Parameters	
		Shoot/ explant.	Shoot length (mm)
		p values	
Medium states	2	0.0166 *	0.0001 **
Sucrose	3	0.0002 **	3.9E-08 **
pH	3	0.3031	0.7994
States*Sucrose	6	0.0764	0.0141 *
States*pH	6	0.3935	0.6044
Sucrose*pH	9	0.0039 **	1.0E-05 **
States*Sucrose*pH	18	0.1520	0.2460
Error	96		
Total	144		

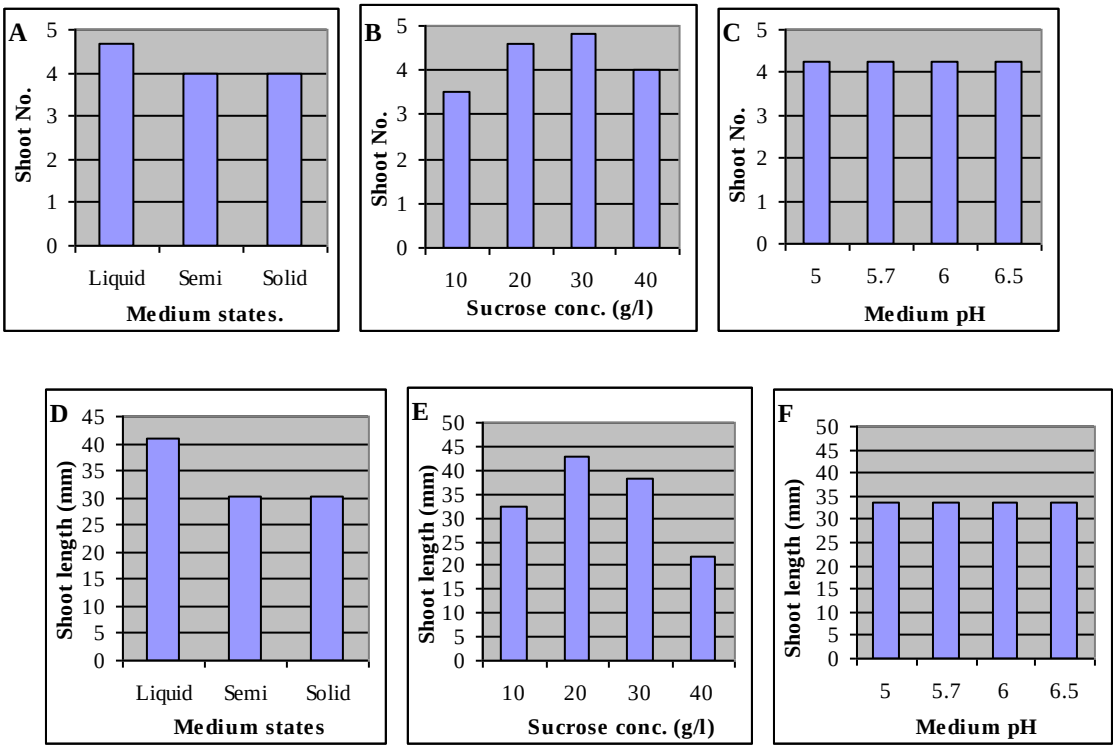


Figure (3.6). Overall effect of medium states, sucrose concentrations and pH adjustments on shoot No.(A, B and C) and shoot length (D, E and F) of Moris pineapple cultured on full strength MS enriched with BAP at 2.0 mg/l for 60 days. The bars of medium states were means of 144 shoots and the bars of sucrose and pH adjustment were means of 108 shoots.

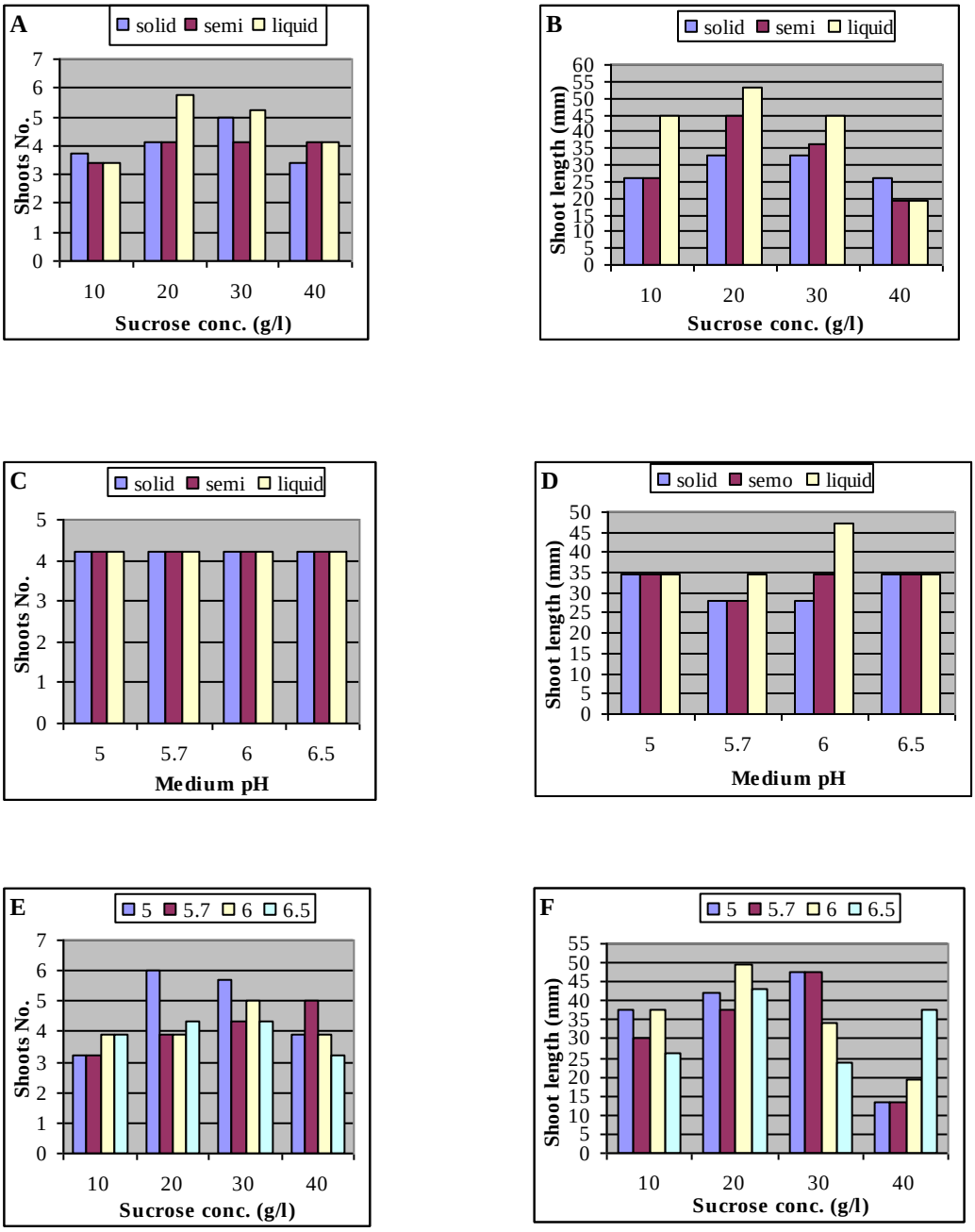


Figure (3.7). *In vitro* shoot formation per explant of Moris pineapple cultured in full strength MS medium enriched with BAP at 2.0 mg/l at different combinations of sucrose concentrations and medium states (A), pH adjustments and medium states (C) and sucrose concentration and pH adjustments (E) and the shoot length (B, D and F) after 60 days of incubation.

Table (3.18). Effect of medium states, sucrose concentrations and pH adjustments on shoot formation and shoot length per explant of Moris pineapple

		Medium states			Medium states		
Sucrose	**pH**	Solid	Semi	Liquid	Solid	Semi	Liquid
		Shoots per explant			Shoot length (mm)		
10	5	3 c	3 c	3 c	23 defghijkl	42 abcdefgh	45 abcdefg
	5.7	4 bc	3 c	3 c	18 ghijkl	22 efghijkl	51 abcd
	6	4 bc	3 c	4 bc	20 fghijkl	28 cdefghijkl	60 ab
	6.5	4 bc	4 bc	4 bc	34 abcdefghij	17 hijkl	28 cdefghijkl
20	5	6 ab	5 abc	7a	36 abcdefghi	43 abcdefg	46 abcdef
	5.7	3 c	3 c	6 ab	32 bcdefghijkl	33 abcdefghijk	47 abcde
	6	4 bc	4 bc	4 bc	33 abcdefghijkl	55 abc	61 a
	6.5	3 c	4 bc	6 ab	29 cdefghijkl	42 abcdefgh	58 ab
30	5	7a	5 abc	5 abc	41 abcdefgh	46 abcdef	56 abc
	5.7	4 bc	4 bc	5 abc	46 abcdef	45 abcdefg	50 ancde
	6	5 abc	4 bc	6 ab	18 ghijkl	38 abcdefghi	46 abcdef
	6.5	4 bc	4 bc	5 abc	26 defghijkl	17 hijkl	28 cdefghijkl
40	5	3 c	5 abc	3 c	22 efghijkl	7 kl	12 jkl
	5.7	5 abc	6 ab	4 bc	14 ijkl	14 ijkl	12 jkl
	6.5	3 c	3 c	6 ab	32 bcdefghijkl	5 l	21 fghijkl
	6	3 c	3 c	4 bc	38 abcdefghi	48 abcde	38 abcdefghi

Data were means of 9 explants cultured on full strength MS medium enriched with BAP at 2.0 mg/l and incubated for 60 days

Means of the same parameters (shoot per explant and shoot length) followed by the same letter were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range Test.

Table (3.19). Estimated cost of multiplication of Moris pineapple at different combinations of sucrose concentrations, pH adjustments and medium states

Sucrose (g/l)	pH	Medium states		
		Solid	semi	liquid
		Cost per shoot (RM)		
10	5	0.33	0.31	0.30
	5.7	0.24	0.31	0.30
	6	0.24	0.31	0.22
	6.5	0.24	0.23	0.22
20	5	0.17	0.19	0.13
	5.7	0.33	0.32	0.15
	6	0.25	0.24	0.23
	6.5	0.33	0.24	0.15
30	5	0.14	0.19	0.19
	5.7	0.25	0.24	0.19
	6	0.20	0.24	0.16
	6.5	0.25	0.24	0.19
40	5	0.34	0.20	0.16
	5.7	0.21	0.17	0.24
	6.5	0.34	0.33	0.16
	6	0.34	0.33	0.24

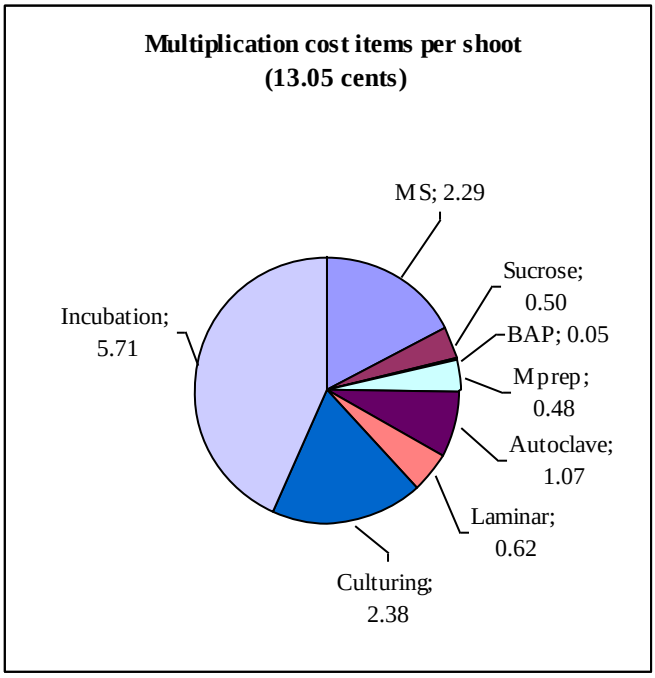


Figure (3.8). Estimated cost items of in vitro multiplication per shoot of Moris pineapple cultured at density of one shoot per culture after incubation for 60 days on full strength liquid MS medium enriched with sucrose at 20 g/l and BAP at 2.0 mg/l and adjusted to pH 5.0. Cost in Malaysian cents
Mprep = (medium preparation).
Culturing (separation and culturing of shoots)
Autoclave, laminar and incubation (electicity cost for operation of autoclave, laminar and incubation room)

Moris pineapple and in development of multiplication medium that keep the activity of these enzymes at its highest level constantly.

3.2.9. Effect of hormone types and concentrations on the multiplication and growth of Moris pineapple shoot on liquid MS medium.

Analysis of variance showed that *in vitro* shoot formation was significantly affected by hormone types, concentrations and interaction of the two factors ($p \leq 0.005$). Overall, BAP and NAA resulted in highest shoot formation (5.7 and 5.9 shoots) and KN in intermediate shoot formation (4.6 shoots) and each of these hormones resulted in shorter shoots (16 mm). Explants treated with IAA produced the fewest (3.8 shoots per explant) but longest (34 mm) shoots (Table, 3.20). Individual comparisons of the combinations of hormones and concentrations showed that the BAP concentrations could be grouped into five different groups. The first and second groups included only one concentration each (2.0 and 3.5 mg/l) and resulted in the highest shoot formation of 8 and 7 shoots respectively. The third group consisted of 3 (BAP at 1.0, 3.0 and 5 mg/l) and the fourth group consisted of 4 (0.5, 2.5, 4.0 and 4.5 mg/l) concentrations each resulted in formation of 5 and 6 shoots per explant respectively. The fifth group consisted of one concentration (1.5 mg/l) and was not different from that to hormone free medium (4 shoots per explant). The shoot formation in generally increased as the BAP concentration increased up to 2.0 mg/l and declined afterwards. None of the BAP concentration resulted in shoot rate less than that of hormone free medium and only one concentration was as effective as hormone free medium (Table, 3.20).

Explants in medium enriched with KN at 1.5, 3.0 and 5.0 mg/l was as effective as hormone free medium (4 shoots per explant) and at concentration of 4.0 and 4.5 mg/l produced less shoots (2 and 3 shoots) than hormone free medium. At the other concentrations (0.5, 1.0, 2.0, 2.5, and 3.5 mg/l) application of KN resulted in more (5 to 6

shoots) shoots than hormone free medium. The highest shoot formation of the explants treated with KN (7 shoots) obtained in medium enriched with KN at 2.5 mg/l. The shoot formation increased as the KN concentrations increased up to 3.0 mg/l and decreased afterwards. The NAA concentrations could be divided into 6 groups. NAA at 3.5 and at 4.5 mg/l resulted in 8 and 9 shoots respectively. NAA at 4.0 and 5.0 mg/l resulted in 7 and NAA at 2.0 mg/l resulted in 6 shoots while NAA at 0.5, 1.5 and 2.5 resulted in formation of 5 shoots. NAA at 1.0 and 3.0 mg/l each resulted in formation of 4 shoots and was not different from hormone free medium. None of the NAA concentration resulted in shoot rate less than hormone free medium and only 2 concentrations was as effective as hormone free while 8 resulted in more shoots than hormone free medium. The proliferation increased as the NAA concentrations increased up to 4.5 mg/l. In fact, the highest proliferation of all treatments (9 shoots) obtained in medium enriched with NAA at 4.5 mg/l. The highest shoot formation of the explants treated with IAA was 5 shoots obtained at 4.5 and 5.0 mg/l. The other concentration resulted in either less (0.5, 2.5, 3.0, 3.5 and 4.0 mg/l) or equal (1.0, 1.5 and 2.0 mg/l) shoots as that of hormone free medium (4 shoots).

The tallest shoot (56 mm) of all treatments obtained in medium enriched with IAA at 2.5 mg/l and the shortest (6 mm) obtained in medium enriched with KN at 4.0 mg/l. Compared to hormone free, presence of BAP, NAA and KN suppressed shoot elongation. All concentrations of BAP and all of the NAA except at 3.0 mg/l and KN except 1.0 mg/l resulted in shoot shorter (two time less) than hormone free medium. On the contrary, IAA at 4 concentrations (low and high) had no effect and at the other 6 (intermediate) promoted the shoot elongation. The shoot length increased as the IAA concentration increased to a peak of 55 mm long at 2.5 mg/l (two times the shoot length in hormone free medium) and then declined afterward.

Using of liquid is advantageous over solid medium. It saved the cost of agar, reduced the laminar operation time (culturing), cleaning of vessels. However, obtaining of highest rate of shoot formation depended on using the optimal hormone type and concentration. BAP applied singly and in combination with NAA and IAA is the most commonly tested hormones for multiplication of pineapple. However, in all of the reported studies, the range of tested concentrations was very narrow (less than 4 different concentrations at the most) and single application was limited to cytokinine type of hormone. The effect of singly applied NAA and IAA was not reported or it may was not tested. The results (Table, 3.20) indicated that different conclusions of which the best hormone and concentration could be made depending on how wide the range of tested concentration was and which concentrations included in that range. Equal shoot formation could be obtained at equal concentration of different hormones and at different concentrations of the same hormone. On other word, depending on the tested concentrations range either one of these three hormones could be claimed better than the others. At equal concentration of 0.5 mg/l and 3.0 mg/l both of KN and NAA produced 5 and 4 shoots respectively. At equal concentration of 5.0 mg/l both of BAP and IAA produced 5 shoots while at 1.0 mg/l both of NAA and IAA produced 4 shoots. At equal concentration of 1.5 mg/l, application of BAP, KN and IAA resulted in formation of 4 shoots. Formation of equal number of shoot was also obtainable using different hormones at different concentrations. Equal rate of 8 shoots was obtained response to two hormones, BAP at 2.0 mg/l and NAA 3.5 mg/l while equal rate of 7 shoots obtained using three type of hormones BAP at 3.5, KN at 2.5 and NAA at 4.0 and 5.0 mg/l. Equal rate of 6 shoots obtained at 4 concentrations of BAP, 2 concentrations of KN and 1 concentration of NAA while 5 shoots obtained at 3 concentrations of BAP, 2 concentrations of KN, 3 concentrations of NAA and 2 concentrations of IAA (Table, 3.20). The shoot formation of

Moris could be induced using cytokinin as well as auxin particularly NAA. In fact at all concentrations, NAA was better than KN and at higher concentrations (3.5 to 5.0 mg/l), NAA was even more effective than BAP (Table, 3 20). Comparison of the results obtained at equal concentration of the different hormones showed that BAP was the best of all hormones at three concentrations, better than KN at 8, better than NAA at 5 concentrations, equal to KN at 1 but less than KN at 1 and less than NAA at 5 concentrations. Similar, KN was better than all hormones at 2 concentrations and NAA better than all at 5, better than KN at 6 and better than BAP at 5 concentrations.

Compared to hormone free medium, out of the 10 concentrations, all of the BAP concentrations except one and all of the NAA except 2 concentrations resulted in more shoot formation (promoted) than that of hormone free medium. KN at 2 concentrations resulted in less (inhibited), at 3 concentrations resulted in equal (no effect) and only at 5 concentrations resulted in more shoots (promoted) than hormone free medium. None of the IAA, on the other hand, resulted in shoot formation higher than that of hormone free medium (promoted) while 5 resulted in less (inhibited) and two equal shoots (no effect) as that of hormone free medium. In other word the BAP hormone was 90 % effective inducer and 10 % neutral and NAA was 80 % inducer and 20 % neutral and both had no inhibition effect. KN was 50 % inducer, 20 % inhibitor and 30 % neutral while IAA was 20 % inducer, 50 % inhibitor and 20 % neutral. Conclusion of which hormone or concentration is the best would vary depend on how wide the range of concentrations and whether that range included the inducer, neutral or the inhibitor level of concentrations.

Using Smooth pineapple and liquid medium, Omokoio *et al.* (2001) found that KN at 4 mg/l was better than BAP, while Fitchet (1990a) reported that KN at 2 mg/l was better than BAP and ZN. On the contrary, Table 3.20 showed that KN at 4 mg/l resulted in the lowest rate (2 shoots) and BAP was better than KN at 2.0 mg/l. Aydieh *et al.* (2000), Almeida *et*

Table (3.20). Effect of hormone types and concentrations on the *in vitro* proliferation and growth of Moris pineapple in liquid full strength MS medium

Conc. (mg/l)	**Hormones types**				
	BAP	KN	NAA	IAA	Average
Shoots per explant					
0	4 cde	4 cde	4 cde	4 cde	4 C
0.5	6 abcd	5 bcde	5 bcde	3 de	4.75 ABC
1	5 bcde	6 abcd	4 cde	4 cde	4.75 ABC
1.5	4 cde	4 cde	5 bcde	4 cde	4.25 C
2	8 ab	5 bcde	6 abcd	4 cde	5.75 AB
2.5	6 abcd	7 abc	5 bcde	3 de	5.25 ABC
3	5 bcde	4 cde	4 cde	3 de	4 C
3.5	7 abc	6 abcd	8 ab	3 de	6A
4	6 abcd	2 e	7 abc	3 de	4.5 BC
4.5	6 abcd	3 de	9a	5 bcde	5.75 AB
5	5 bcde	4 cde	7 abc	5 bcde	5.25 ABC
Average	5.7 A	4.6 B	5.9 A	3.8 C	
Shoot length (mm)					
0	27 cdefgh	27 cdefgh	27 cdefgh	27 cdefgh	27 A
0.5	13 ghi	17 fghi	14 fghi	22 cdefghi	16.5 CD
1	11 ghi	32 bcdef	20 defghi	25 cdefgh	22 ABCD
1.5	17 fghi	23 cdefghi	17 fghi	35 bcde	23 ABC
2	11 ghi	17 fghi	12 ghi	29 cdefg	17.2 BCD
2.5	14 fghi	10 hi	14 fghi	56 a	23.5 ABC
3	14 fghi	18 efghi	23 cdefghi	46 ab	25.25 AB
3.5	9 hi	12 ghi	11 ghi	29 cdefg	15.25 CD
4	12 ghi	6 i	12 ghi	25 cdefgh	13.75 D
4.5	11 ghi	9 hi	12 ghi	36 bcd	17 BCD
5	18 efghi	11 ghi	14 fghi	39 bc	20.5 ABCD
Average	14.3 B	16.5 B	15.9 B	33.5 A	

Data were mean of 6 explants cultured at density of two explants on culture tubes containing 10 ml of liquid full strength MS medium supplemented with sucrose at 20 g/l and incubated for 60 days under constant temperature and 16 hour of light provided by cool white floresence lamps.
Mean followed by same letters were not significantly different according to Duncan Multiple Range test at $p \leq 0.05$

Table (3.21). Estimated variable cost per shoot during multiplication of Moris pineapple on liquid MS medium enriched with different hormones at different concentrations.

Conc	Hormone types			
(mg/l)	BAP	KN	NAA	IAA
	Cost per shoot (RM)			
0	0.11	0.11	0.11	0.11
0.5	0.08	0.09	0.09	0.15
1	0.09	0.08	0.11	0.11
1.5	0.11	0.11	0.09	0.11
2	0.06	0.09	0.08	0.11
2.5	0.08	0.07	0.09	0.15
3	0.09	0.11	0.11	0.15
3.5	0.07	0.08	0.06	0.15
4	0.08	0.23	0.07	0.15
4.5	0.08	0.15	0.05	0.09
5	0.09	0.11	0.07	0.09

Multiplication medium was 10 ml of liquid full strength MS medium enriched with sucrose at 20 g/l and different hormone treatment. The cultures incubated for 60 days under constant temperature and 16 hour of light provided by cool white floresence lamps.
RM. Malaysian Ringgit. (1 $ = RM 3.25) and the estimated cost included only the variable cost items (Culture tubes, MS, sucrose, hormones, labor wages and electricity for operating laminar, autoclave and incubation room).

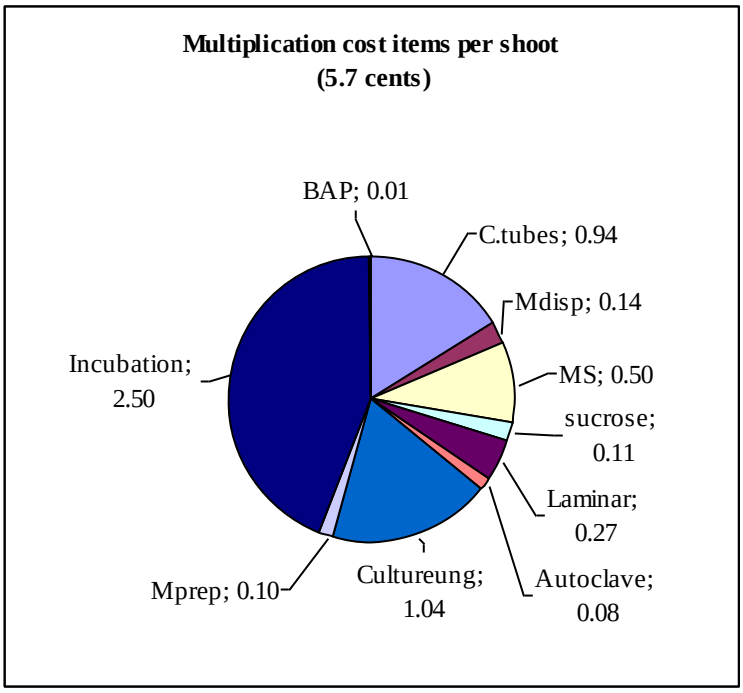


Figure (3.9). Estimated values of multiplication cost item per shoot of Moris pineapple cultured at density of 2 explants per 10 ml of full strength liquid MS enriched with sucrose at 20 g/l and BAP at 2 mg/l after 60 days of incubation
Mprep= (medium preparation); Mdisp= (medium dispensing), Culturing (separation and culturing of shoots under laminar).
Autoclave, Laminar, Incubation (electricity cost for operation of autoclave, laminar and incubation room)

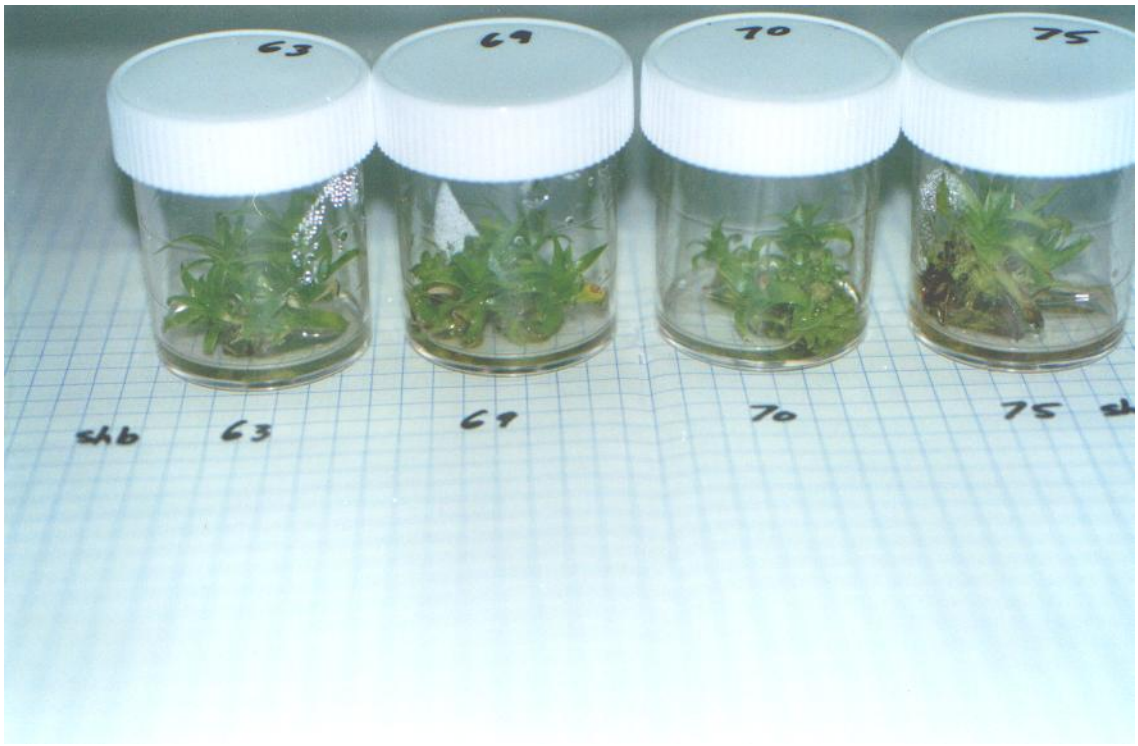


Plate (3.4) Multiplication of Moris pineapple in 10 of liquid full strength MS enriched with different BAP concentrations 1.0, 2.0, 3.0 and 3.5 mg/l (culture tubes No. 63, 64, 70 and 75 respectively) after 60 days of incubation.

al. (2002) and Fernando (1986) tested single application of BAP for Queen, Perola and Mauritius pineapple and recommended different concentrations of BAP, 2.0, 1.5 and 1.0 mg/l respectively. For *in vitro* multiplication of Moris in liquid medium, BAP at 2.0 mg/l was the best of 10 concentrations of BAP, KN and IAA. Both of KN and IAA were not suitable hormones for proliferation of Moris in liquid full strength MS medium. But, IAA promoted elongation substantially (doubled the shoot length). The highest shoot formation (9 shoots) and lowest cost per shoot (5 cents) obtained using NAA at 4.5 mg/l (Table, 3.20 and 3.21). BAP at 2.0 mg/l was the second best treatment with 8 shoots per explant and cost of 6 cents per shoot and would be favored over using NAA at high concentrations (4.5 mg/l). IAA at 2.5 mg/l resulted in the tallest shoots (56 mm) but tripled the cost of shoots (15 cents). Using of KN at 4.0 mg/l resulted in the lowest shoot formation and the highest cost per shoot (23 cents). In all of the NAA and BAP treated explants, the cost was less than hormone free medium while inclusion of IAA resulted in higher cost per shoot than hormone free medium. Dividing the cost into the various cost items (Figure, 3.9) indicated that the electricity of the incubation room was the major cost factor (45 %) of micropropagation followed by cost of labor (22 %), culture tubes (16 %) and MS (9 %). Adopting of outdoor incubation (Be and Debergh, 2006) and reusing of culture tubes over multiplication could substantially lower the cost below the estimated 6 cents per shoot.

3.2.10. Effect of explants density and size on *in vitro* proliferation and growth of Moris pineapple

Explants density had no independent direct effect on the shoot formation per explant, but interacted significantly with explants size. The shoot length, on the other hand, was under direct and indirect effect (via interaction with the explant size) of explants density. Overall, each explant produced 5 shoots whether it was cultured individually or at density of 2, 3, 4 and 5 explants per culture tube (Figure, 3.10A). Using of 1 explant per culture

resulted in shortest shoots (10 mm), but the shoot length increased to 16 mm long as the explants density increased to 3 explants per culture and remained stable at density of 4 and 5 explants per culture. However, contrary to its equal effect on shoot formation per explant, different explants density resulted in substantial different in the expected total shoots per one liter of medium. The total shoots increased from 500 at density of one to 2180 shoots at density of four and five explants per culture (Figure, 3.10C). On the other hand, explants size had significant independent effect on both parameters (shoot formation and length) and via interaction with explant density. The shoot formation and shoot length increased as the explants size increased (Figure, 3.10B). The lowest proliferation of 4 shoots per explant obtained when shorter explants (5 and 10 mm long) were used and the highest proliferation per explant (6 shoots) obtained from longer explants (15, 20 and 25 mm long). Every 5 mm increase in explants size, on the other hand, resulted in significant increase in the shoot length. The shoot total per liter of medium increased from 1113 when the explants was 5 mm long to 1747 shoots when the explants was 15 and 20 mm long, but declined to 1520 shoots when 25 mm long explants was used.

Comparing the shoot formation and shoot length per explants of equal and different size at different explant density revealed that the explant shoot formation capacity depended on its size and explants density per culture (Figure, 3.11A). The longer explants had higher shoot formation capacity than shorter explants. The lowest shoot formation was 2 and 3 shoots per explant obtained from the 5 and 10 mm long explants and the highest rate was 8.3 shoots per explants obtained from the 25 mm long explants, all cultured at density of one explant per culture. However, the explants density effect on the shoot formation followed three different pattern varied according to the explant size. The shoot formation capacity of equal explant size could either be improved or suppressed by the explant density. The highest shoot formation of the 15 and 20 ml long explants (7.0 and 6.7

shoots respectively) obtained at density of four and the lowest shoot formation (4.3 and 4.7 shoots) obtained at density of five explants per culture. The highest proliferation of the 10 mm-long explants (5.7 shoots) obtained at density of two explants per culture. Then, the explants lost gradually its shoot formation capability and the rate declined to 3.7 shoots per explants at density of five explants. The formation pattern in case of the 5 and 25 mm-long explants were amazingly opposite (Figure, 3.11A). The shoot formation of the 5 mm long explants increased from 2.7 shoots to 3.7 while that of the 25 mm long explants decreased from 8.3 to 3.7 shoots as the explants density increased from one to three explants per culture. Compared to density of 3 explants, increasing the density to 4 and 5 explants per culture did not effect the proliferation potential of the 5 mm long explants but improved the potential of the 25 mm long explants. The 5 mm-long explants maintained its shoot formation potential while the 25 mm explants regained some capability for shoot formation. The 25 mm long explants produced more shoots than that obtained at density of three, but was less than that obtained at density of one and two explants per culture.

The effect of explants density per culture on the expected total of shoots per liter of medium (Figure, 3.11B) followed also three different patterns. The relationship between the explants density and total shoots of the 5 mm long explants were almost linear. The total shoots per liter increased from 267 shoots at density of one to a maximum of 2000 shoots at density of five explants. For the 10 and 25 mm long explants, the total increased as the density increased up to five. However, in case of 10 mm, the increase in total was rapid as the density increased to three explants but slowed down at density of four and five while the opposite was true in case of 25 mm long explants. Slow increase in total as the density increased to 3 explants but the total shoots production accelerated markedly afterward. A minimum of 233 and 833 shoots per liter at density of one and a maximum of 1833 and 2667 shoots at density of five explants were obtained from the 10 and 25 mm

long explants respectively. The curves of total shoots obtained from the 15 and 20 mm long explants were almost identical. The lowest shoot total of both explants were 500 and 667 shoots obtained at density of one explant per culture and the total increased as the density increased to reach highest total of 2800 and 2667 shoots at density of four and then declined to 2167 and 2333 shoots at density of five explants per culture. According to both of shoot formation rate per explant (Figure, 3.11A) and total per one liter (Figure, 3.12B), 15 and 20 mm-long explants should be used at density of four explants per culture. For the 25, 10 and 5-mm long explants, the shoot formation rate per explant suggested a density of one, two and three respectively as the best choice. However, according to total per liter, a density of five explants per culture resulted in the highest total. Similar to its effect on shoot rate and total shoot, the effect of explants density on shoot length varied depending on the explants size (Figure, 3.11C). When the 15 and 25 mm long explants were used, the shoot length increased respectively from 12 and 14 mm to 16 and 22 mm as the density increased from one to four explants and then decreased to 13 and 19 mm at density of five. In case of the 5, 10 and 20 mm long explants, the shoot length generally was increased as the density increased up to five explants per culture.

Table 3.22 and figure 3.12 showed that the effect of the explants density and size was not limited to average shoots formation per explant and total shoots per liter of medium, but also extended to the frequency of shoots of different length per liter of medium. Different densities that may have equal effect in term of total shoots per liter could result in different frequency of shoots of different length and vice versa. For instance, overall explants size, density of four and five explants per culture (Table, 3.22) resulted in statistically equal total of 2160 and 2200 shoots per liter respectively. But the shoots were of different size. The difference in the total shoots within the shoot length range of 6-10 mm (400 and 533 shoots), length range of 11-15 mm (480 and 467 shoots) and length

range of ≥ 26 mm long (267 and 233 shoots) obtained from both densities (four and five explant per culture) per liter were also statistically equal. However, while the total shoots per liter within length range of < 5 mm (467 shoots) and length range of 16-20 mm (367 shoots) at density of 5 explants were significantly higher than the 400 and 347 shoots obtained at density of 4 explants per culture, the total shoots per liter within the length range of 21- 25 mm long obtained at density of 5 (133 shoots) were significantly less than that (267 shoots) produced at density of 4 explants per culture. Using of 1, 2 and 3 explants per culture resulted in significant different total of 500, 1013 and 1560 shoots per liter and also in significant different total of shoots that were shorter than 5 mm (160, 240 and 300 shoots), shoots that were between 6-10 mm long (107, 213 and 400 shoots), between 11-15 mm (93, 227 and 380 shoots) and between 16- 20 mm long (67, 160 and 240 shoots). However, the different in total shoot that were between 21- 25 mm long (40, 107 and 80 shoots) and shoots that were longer than 26 mm (33, 67 and 120 shoots) was not significant.

The total and frequency of shoots of different length per one liter of medium was also affected by the size of the explants (Table, 3.22). The highest total of 1747 and 1680 shoots per liter was obtained when 20 and 15 mm -long explants were used respectively and the lowest total of 1113 shoots obtained from the 5 mm-long explants. The frequency of shoots shorter than 10 mm long was not affected by the explant size. At each explant size, an average of 313.4 and 330.6 shoots of the total shoots per liter were within the shoot length range < 5 and 6- 10 mm long respectively. However, the total of shoots per liter of medium within the shoot length range of 11- 15; 16- 20; 21 -25 and > 26 mm long were significantly affected by the explants size. Using of longer explants increased the possibility of producing shoots longer than 25 mm. At 5, 10 and 15 mm long explants, the

total of shoots longer than 25 mm were 73, 40 and 113 shoots, about 7 %, 3 % and 8 % of the total of all shoots per liter while using of 20 and 25 mm long explants increased the amount of shoots longer than 26 mm to 227 and 267 shoots respectively. That is about 13 and 18 % of total shoots. This significant different in shoot formation per explant, total shoots per liter and frequency of shoots of different shoot length per liter of medium between explants of different size at different explants density would have great impact in the total shoots produced in the subsequent cycle of multiplication. Generally, the majority of shoots (66 %) were within the range 5 to 15 mm long and few within range 16 to 30 mm long. Overall, the percentage of the shoots ≤ 5; 6-10; 11-15; 16-20; 21-25 and ≥ 26 mm long were 21; 22; 22; 16; 8 and 10 % of the total shoots per one liter of medium respectively (Figure, 3.13).

Estimated cost per shoot (Table, 3.23) shows that the longer the explants and the higher the density the lower the cost per shoot. Using of 10 mm long explants at density of 1 explant per culture resulted in the highest cost per shoot (45 cents) while the lowest cost of 3 cents per shoots obtained using 15 mm long explants at density of 4 explants per culture. Increasing the density of the 10 mm long explants to 5 explants per culture reduced the cost from 45 to 5 cents per shoot and using of longer explants (15, 20 and 25 mm long) reduced the cost from 45 cents to 18, 13 and 12 cents. Increasing the density of the 5, 10, 15, 20 and 25 mm long explants from 1 to 5 explants per culture reduced the cost per shoot from 32, 45, 18, 12 and 12 cents to 4 cents and increasing the explants size at density of 1, 2, 3, 4 and 5 explants from 5 to 25 mm long explants decreased the cost per shoot from 32, 18, 7, 6 and 4 cents to 12, 8, 8, 4 and 4 cents respectively. It is worth noting that according to shoot formation rate per explant the best treatment was 25-long explants at density of 1 explant per culture (8.3 shoots per explant). But this treatment resulted in lower total (700

shoots per liter) and higher cost per shoot (12 cents). Using of 15 long explants resulted in highest total per liter (2800 shoots) and lowest cost per shoot (3 cents) but was the second best treatment (7 shoots per explant) in term of shoot formation per explant. Different total shoots per one liter of medium could be produced at equal cost of 3 cent per shoot at two combinations of explants density and size (15 and 20 mm long explants at density of 4 explants per culture) and at equal cost of 4 cent per shoot at 7 different combinations of explants size and density (Table, 3.23). Since in all treatments the total cost per one liter of medium was RM 90.85, treatment with higher total would be favored over those with low total shoots per liter. It clear that evaluation of different treatments by comparing of shoot formation rate per explant as it most commonly done during investigation of *in vitro* multiplication is not enough for management and commercial production of propagules. Partitioning of the cost into the different cost item indicated that the cost of electricity bill during incubation period was the most cost causing factors (44 %) followed by labor wages for culturing of the explants (22 %) and cost of culture tubes (17 %).and MS powder (9 %). Lowering the strength of medium and using of less medium volume per culture may reduce the cost of medium while the cost of incubation may reduce by using different incubation periods.

At experimental level, few selected shoots of specific size are usually used for testing the effect of certain factors on multiplication. The results of this study supported the selective use of 15 to 20 mm long shoots as the most competent shoot for experimental purposes. Overall density, the 15-20 mm long shoots resulted in highest shoot formation per explant, highest total per liter and intermediate shoot length. However, in commercial mass production all shoots produced in one multiplication cycle would be reused for the next cycle. At any multiplication cycle shoots of different length are produced. Assuming of equal shoot formation capacity of shoots of different size and similar responses to

different explants density, simplify the culturing procedure but at expense of optimum multiplication rate. Individual comparison of explants size and density combinations (Figure, 3.11A) showed that explants of different size responded differently to different explants density. The shoot formation of the longest explants (25 mm) decreased as the density increased while the shoot formation of the shortest explants (5 mm) increased as the density increased to 3 explants and remained stable afterwards. The shoot formation of the 10 mm long shoots increased up to density of 2 explants and that of 15 and 20 mm long increased up to density of 4 explants per culture but decreased afterwards. That is for optimal multiplication, the 25 mm long explants should be cultured individually, the 10 mm long at density of 2, the 15 and 20 mm long at density of 4 and the 5 mm long at density of 3 explants per culture. In almost all of the reported micropropagation studies of pineapple, the attention was focused on optimization of hormones and medium states. Other factors as explants length and density are not only important as shoot formation effecting factors but also very crucial for cost estimation and management yet were totally ignored. Explants density was compared as clusters rather than single intact shoots in a bioreactor system (Escalona, *et al*. 1999) and together with incubation period induced significant effect on the frequency of shoots of different lengths. Using of different explants size induced significant different effect on shoot formation of other plants such as *Lilium longiflorum* (Nhut *et al*., 2001), rose (Salehi and Khosh-Khui, 1997; Acker and Scholten, 1995) and *Populus* spp (Douglas, 1984), *Petunia* (Beck and Camper, 1991). Similar, density of explants per culture induced significant different on rate of shoot formation of potato (Sarkar, *et al*. 1997), *Alstromeria* (Bond and Alderson, 1993) and *Populus alba* x *P. grandidentata* (Chun *et al*. 1986).

Total shoots per liter were not used before as an assessment parameter, however, it is more important and practical than the rate of shoot formation. Overall explants size,

different explants density resulted in equal shoot formation rate per explants (Figure, 3.10A), but higher density resulted in higher total shoot per liter (Figure, 3.10B) and longer shoots (Figure, 3.10C). The total shoot per liter increased from 500 to 2160 shoots and the shoot length increased from 10 mm to 15 mm as the explants density increased from one to four explants. Total shoots per unit of medium (liter) is very suitable index that could reflect the efficient use of all production units. Knowing the amount of total shoots at one multiplication cycle, the number of vessels, shelving space and working hour of the next cycle could safely estimated and used for cost analysis. According to rate per explant, the 25 and the 10 mm long explants should be cultured at density of 1 and 2 explants per culture. The rate per explant of the 25 mm long at density of 1 was two times higher and that of the 10 mm long was 1.5 higher than the rate at density of 5 and 2 explants per culture respectively. However, the highest total per liter was obtained when the 25 and the 10 mm long shoots were cultured at density of 5 and 4 shoots per culture respectively. That is the using of density of 5 reduced the cost of jars and medium by 5 times and would result in three times cheaper shoots than at density of 1 shoots per culture. For the 15 and 20 mm long explants, the highest rate per explant and highest total per liter were obtained at density of 4 explants per culture. Using of the highest possible density would reduce the total volume of medium, number of culture vessels as well as the shelving space and labor working hours and hence the cost of production as total and as cost per shoot. Arinaitwe *et al.* (2000) suggested use of total number of shoots produced per liter of medium for assessment of cost effectiveness of different cytokinins for different plants.

The most common parameter of assessment of *in vitro* multiplication is the shoot formation rate per explant. Shoot lengths were in most time not reported and in few cases was given as average of all shoots of that cycle in response to specific treatments. Although, the overall average of shoot rate and length give an indication of whether a

treatment promoted or suppressed shoot formation and length, it ignored the difference in the frequency of shoot size between the different treatments. Total per liter and total at each cycle of multiplication is essential for better management and planning of commercial micropropagation. However, estimation of total of shoots without taking in account the different shoot length and their frequency and the effect of shoot length and shoots density per culture on the shoot formation capacity would not reflect precise figure. Using 10-L bioreactors, Escalona, *et al*. (1999) reported that different density and incubation periods resulted in significant differences in the frequency of pineapple shoots of different lengths and that difference had significant impact on the subsequent *ex vitro* rooting capability and out door surviving of the obtained shoots. Significant different in the frequency of shoot length were also reported for papaya (Miller and Drew, 1990) and apple rootstock M4 (Dunstan *et al*., 1985). Since the final goal of micropropagation study is mass production of propagules and that would means using of all shoot produced in one multiplication cycle for the next cycle, the effect of size and density need to be addressed and other parameters beside the rate per explant should be included in the assessment of the results. This study (Table, 3.22; Figure, 3.12) showed that the total shoot per liter were of different length. Overall, 21; 22; 22; 16; 8 and 10 % of the total shoots per liter were ≤ 5; 6-10; 11-15; 16-20; 21-25 and ≥26 mm long respectively. It is important that shoots of different shoot length be cultured at the optimal explants density according to the purpose of the experiment. For physiological study, explant should be cultured at the density that resulted in the highest rate per explant (Figure, 3.11A). However, for commercial production the explant should be culture at the density which would result in the highest total shoots per liter (Figure, 3.11B).

The effect of different explants density and explants size on the shoot formation could attribute to nutrient competition and number of axillary buds per explant. Assuming that shorter explants have less axillaries bud and would use less nutrients than longer explants, competition for nutrient could to some extent explain the different in rate. Figure 3.11A showed that at any explants density, longer explants produced more shoot (more axillaries) than shorter explants. The shoot formation of shorter explants (5, 10 and 15 mm long) increased as the density increased up to 3 explants per culture while the shoot formation of longer explants (20 and 25 mm long) decreased. Longer explants used more nutrients than shorter ones and the amount of nutrient used by five of 5 mm long explants might be less than that used by two of 25 mm long explants. The nutrient content of 10 ml of MS medium could be more than that required for 3 short explants but less than that required for 2 long explants. Stability, gradual and sharp decline of rate in case of 5, 10 and15 mm long explants that were cultured at density of more than three explants supported the nutrient competition. However, formation of more shoots at density of 5 than at density of 4 and 3 from the 25 mm long explants suggested that in culture having more than 3 explants, other factor beside competition for nutrient was involved in shoot formation. Konan *et al*. (2007) reported that the rooting of oil palm shoots in a single culture vessel was under affect of coupling factor related to differences in the explants size. That is in a single culture vessel, rooting of one explant affected the rooting of the others and mixing longer explants with shorter ones improved the rooting of all explants within the culture. In this study, mixing of explants of different sizes per single culture tube was not tested. However, the difference in shoot formation rate among the explants of different size and their different responses to explants density per culture indicated that the shoot formations of Moris pineapple were under coupling effect. The effect of different explants density and explants size on the shoot length could attribute to competition for light. Longer explants and higher

density created crowdness, obscured light and make elongation proceeds faster. Figure 3.11C showed that at any density, longer explants produced longer shoots and the length increased as the density (crowdness) increased. Length of shoot is important for *ex vitro* rooting and successful acclimatization of pineapple (Escalona *et al*, 1999). To improve the shoot length, both Escalona *et al*. (1999) and Firoozabady and Gutterson (2003) suggested an elongation stage of one month in which at each two weeks the shoots subjected to different hormone treatment. Combination of higher explants density with GA_3 treatment might result in substantial improvement in the shoot elongation. To obtain longer shoots, it is advisable that during the last cycle of multiplication just before the shoots transfer to rooting stage, the 10 and 20 mm long shoots cultured at density of 5 explants per culture and the 15 and 25 mm long shoots cultured at density of 4 explants while the 5 mm long shoots cultured at density of 3 explants per culture (Figure, 3.11C). In conclusion, although optimization of hormones and medium states are very important for proliferation studies, optimization of the system require selection of the best explants density for the shoots of different length. In addition, besides comparing averages shoot rate and length per explant, the total shoot production, the frequency of different shoots of different length per liter of medium and the cost per shoot should also be considered for assessment of different multiplication treatments.

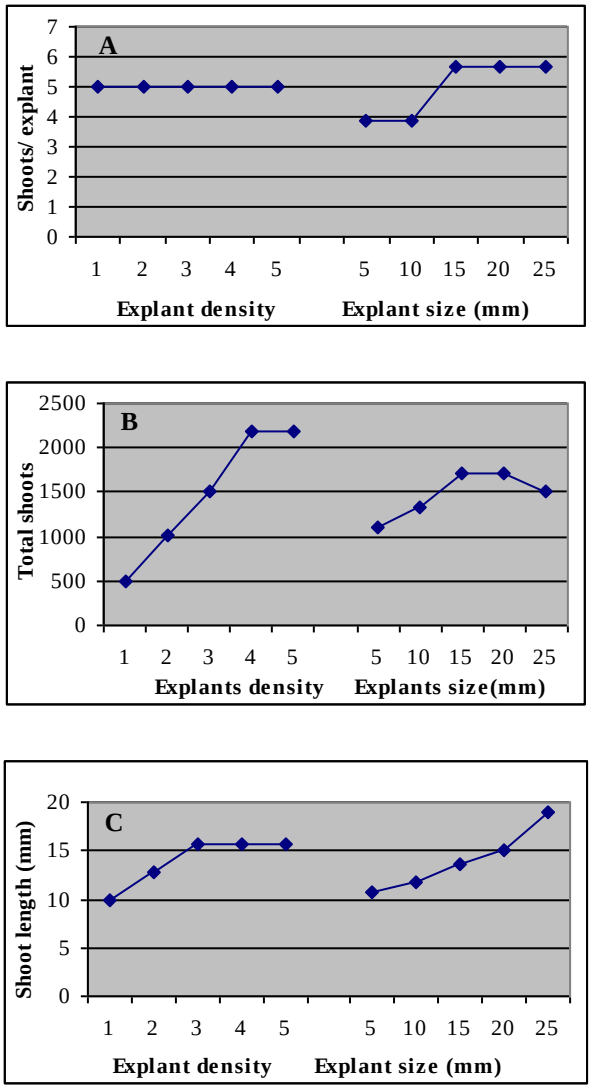


Figure (3.10). Overall effect of explants size and explants density on the shoot formation rate (A) total shoots per liter of medium (B) and shoot length (C) of Moris pineapple cultured on 10 ml of liquid full strength MS medium enriched with sucrose at 20 g/l and BAP at 2.0 mg/l and incubated for 60 days

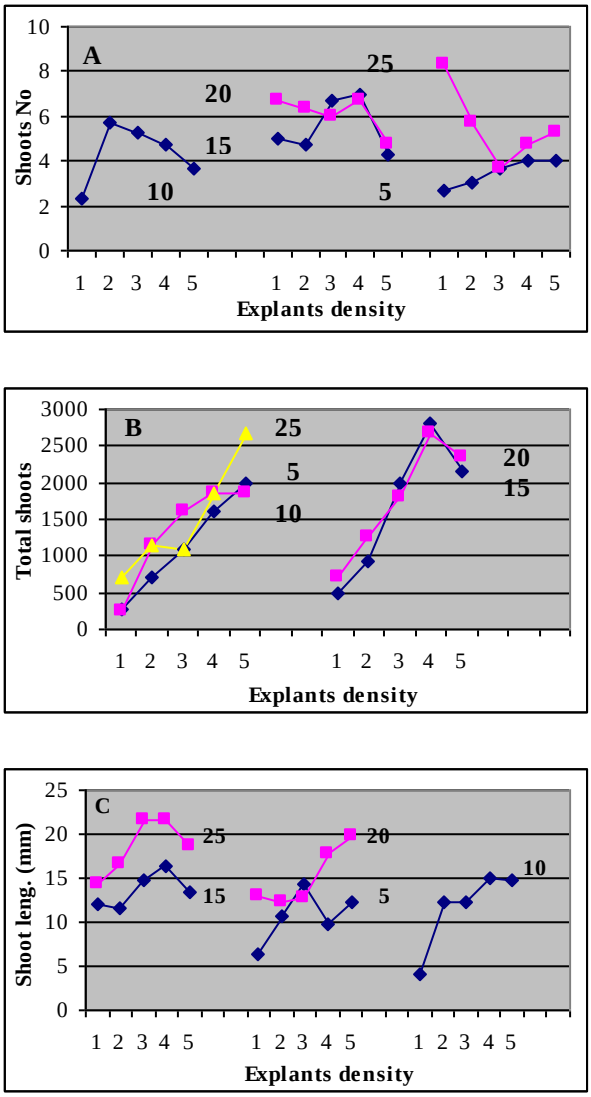


Figure (3.11). Effect of different combinations of explants size and density on shoot formation rate (A) total shoots per liter of medium (B) and shoot length average (C) of Moris pineapple cultured in 10 ml of liquid full strength MS medium enriched with sucrose at 20 g/l and BAP at 2.0 mg/l and incubated for 60 days

Table (3.22). Effect of explants density and size on the total shoots and total of shoots of different length Moris pineapple per liter of static liquid full strength MS medium enriched with sucrose at 20 g/l and BAP at 2.0 mg/l after 60 days of incubation.

Factors	Shoot length range (mm)						
	< 5	6- 10	11- 15	16- 20	20- 25	> 26	Total
	Total per liter						
Explants density							
1	160 d	107 b	93 c	67 d	40 b	33 b	500 D
2	240 cd	213 b	227 b	160 cd	107 b	67 b	1013.3 C
3	300 bc	400 a	380 a	240 bc	80 b	120 b	1520 B
4	400 ab	400 a	480 a	347 ab	267 a	267 a	2160 A
5	467 a	533 a	467 a	367 a	133 b	233 a	2200 A
Explants size (mm)							
5	273 ns	293 ns	273 ab	200 b	0 b	73 b	1113.3 C
10	353 ns	293 ns	247 b	220 ab	180 a	40 b	1333.3 BC
15	347 ns	340 ns	387 a	333 a	160 a	113 b	1680 A
20	347 ns	407 ns	373 a	240 ab	153 a	227 a	1746.7 A
25	247 ns	320 ns	367 a	187 b	133 a	267 a	1520 AB

Total of the same column followed by same letters were not significant as tested by Duncan Multiple Range Test at $p \leq 0.05$.

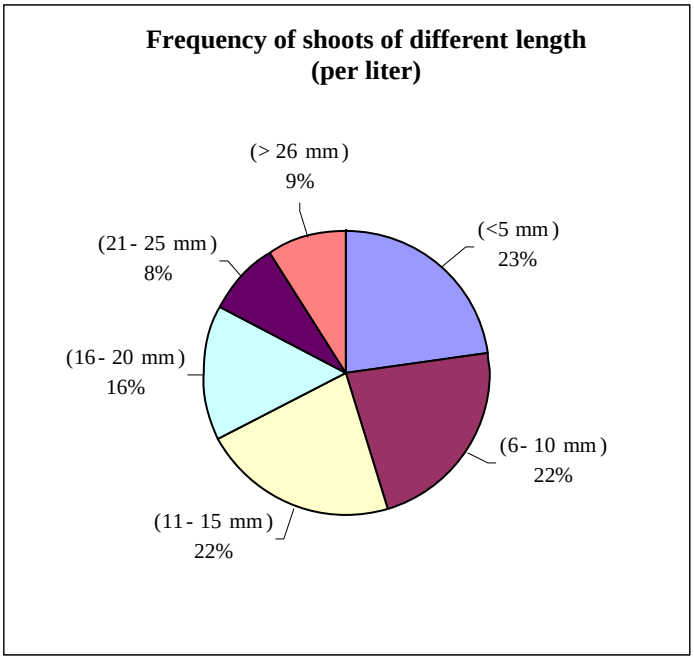


Figure (3.12). Frequency of shoots of different length of Moris pineapple per one liter of staic liquid full strength MS medium enriched with sucrose at 20 g/l and BAP at 2.0 mg/l and adjusted to pH 5.0 after 60 days of incubation.

Table (3.23). Effect of explants size and density on the estimated cost per shoot of Moris pineapple cultured on static liquid full strength MS medium enriched with sucrose at 20 g/l and BAP at 2.0 mg/l for 60 days.

Expl. Size	**Explants density**				
(mm)	1	2	3	4	5
	Total/shoots per liter				
5	266.7 jk	600 hijk	1100 ghi	1600 defg	2000 bcd
10	233.3 k	1133.3 fghi	1600 defg	1866.7 cde	1833.3 cde
15	500 ijk	933.3 ghij	2000 bcd	2800 a	2166.7 abcd
20	666.7 hijk	1266.7 efgh	1800 cdef	2666.7 ab	2333.3 abc
25	833.3 hijk	1133.3 fghi	1100 ghi	1866.7 cde	2666.7 ab
	Cost per shoot (RM)				
5	0.32	0.18	0.07	0.06	0.04
10	0.45	0.08	0.06	0.04	0.05
15	0.18	0.09	0.04	0.03	0.04
20	0.13	0.08	0.05	0.03	0.04
25	0.12	0.08	0.08	0.04	0.04

RM. Ringgit Malaysia (1.0 USA $ = RM 3.25).
Cost included variable cost items only (Culture tubes, Medium, Sucrose, Hormone, Electricity for operating autoclave, laminar and incubation room and labor wages).

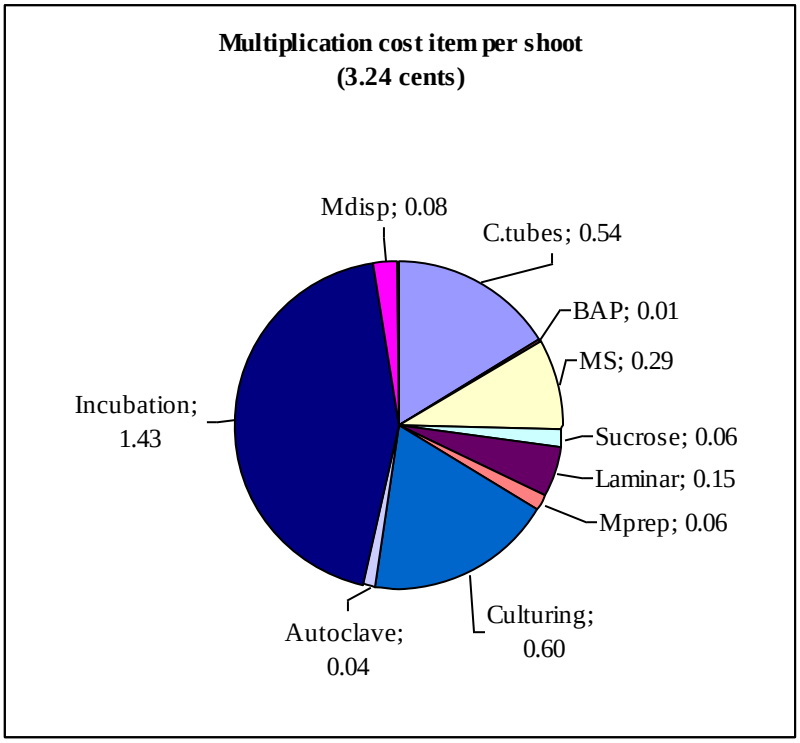


Figure (3.13). Multiplication cost items per shoot of Moris pineapple cultured at density of 4 explants on 10 ml of liquid full strength MS medium enriched with sucrose at 20 g/l and BAP at 2.0 mg/l, adjusted to pH 5.0 and incubated for 60 days. Medium was autoclaved in glass bottle and dispensed in sterilized culture tubes under laminar
Mprep. = (Medium preparation); Mdisp. = (Medium dispensing), Ctube.= (Culture tubes).
Culturing (separation and culturing of shoots under laminar)
Laminar, Autoclave, Incubation (electricity cost for operating laminar, autoclave and incubation room).

3.2.11. Effect of medium volumes and explants density per culture on the multiplication and growth of Morris pineapple

Of all combinations of explants density and medium volumes per culture (Table, 3.24), the highest shoot formation per explant (13 shoots) obtained when one explant was cultured in 10 ml of medium and the lowest (3 shoots) obtained when 4 explants were cultured in 5, 6 and 7 ml of medium. The longest shoots (19.7 mm), on the other hand, obtained when two explants were cultured in 7 ml of medium and the shortest shoots (8.7 and 9.3 mm) when one explant was cultured in 10 ml and 4 explants cultured in 3 ml and 11 ml of medium. However, at each explants density there was different optimal volume of medium per culture and at each medium volume there was different optimal explants density. At fixed volume of 3, 5, 7 and 8 ml of medium per culture the highest shoot formation was 11, 8, 8, and 11 shoots per explant obtained at density of one explant per culture and the lowest shoot formation was 4, 3, 3 and 5 shoots obtained at density of four explants. Dispensing the medium at 10 ml per culture, the highest shoot formation was 13 shoots obtained at density of one explant and the lowest was 5 shoots obtained at density of two while in cultures containing 4, 6 and 12 ml the highest shoot formation was 10, 10 and 11 shoots per explant obtained at density of 3 explants per culture and the lowest shoot formation was 5, 3 and 4 shoots obtained at density four explants per culture. In cultures containing 11 ml of medium the highest shoot formation was 10 shoots per explant obtained at density of two explants per culture and the lowest was 4 shoots obtained at density of four explants. In cultures containing 9 ml, the highest shoot formation was 11 shoots per explant obtained at two different explants density (one and three explants per cultures) and the lowest was 6 shoots obtained also at two different explants density (two and four explants per culture).

Similar, at fixed density of one explant, the highest shoot formation was 13 shoots obtained at 10 ml of medium per culture and the lowest was 6 shoots obtained at 4 ml per cultures. At density of two, the highest shoot formation was 10 shoots per explants obtained at 11 ml and the lowest was 5 shoots per explant obtained at three different volume of medium (6, 7 and 10 ml) per culture. At density of three, the highest shoot formation was 11 shoots obtained at two different volume of medium (9 and 12 ml) and lowest was 5 shoots obtained one medium volume (7 ml per culture). At density of four explants, the highest shoot formation was 7 shoots obtained at 10 ml and the lowest was 3 shoots obtained at three different volumes of medium (5, 6 and 7 ml) per culture (Table, 3 24). In other words, dispensing the medium at volume improper for the explant density and using of explants density improper for the medium volume per culture is just like blocking of 50 % of the explant capacity for shoot formation before the experiment started.

Comparing the effect of explants density on shoot formation at each medium volume with the shoot formation average overall medium volumes indicated that the explants density effect on shoot formation capacity of the explants followed three different patterns and the medium could overcome or aggravated the inhibitory effect of the explants density depending on the medium volume dispensed per culture. In cultures containing 4 and 12 ml of medium, the shoot formation increased from 6 and 9 shoots to 10 and 11 shoots per explant as the explants density increased from one to three explants per culture and the shoot formation per explant was higher than the average of shoot formation overall medium volumes per culture (Figure, 3.14A). That is, these medium volumes overcome and reverse the inhibition effect of the explants density in the shoot formation process. On the contrary, in cultures containing 3, 5, 7 and 8 ml of medium (Figure, 3.14B), the shoot formation decreased from 11, 8, 11 and 8 shoots to 4, 3, 3 and 5 shoots per explant as the density increased from one to four explants per culture and the shoot formation per explant

were lower than the overall density average. That is, these medium volumes aggravated the inhibition effect of higher explants density on the shoot formation. Dispensing the medium at 6, 9 and 10 ml per culture resulted in different shoot formation pattern (Figure, 3.14C). High shoot formation at density of one and three explants and low shoot formation at density of two and four explants per culture but the shoot formation pattern was similar to that of the general effect of explants density on shoot formation.

The shoot length was also affected by proper combination of explants density and medium volumes per culture. At each explants density there was different optimal volume of medium per culture and vice versa. At fixed volume of 4 and 7 ml of the medium per culture, the longest shoots was 15 and 19.7 mm respectively both obtained at density of two explants per culture and the shortest shoots was 11 mm obtained at density of four explants per 4 ml and one explants per 7 ml of medium. At fixed volume of 11 and 12 ml of medium, the longest shoots was 11.7 and 16.7 mm respectively obtained at density of three explants and the shortest was 9.3 and 10 mm obtained at density of four explants per 11 ml and one explant in 12 ml of medium. In cultures containing 8 and 10 ml of medium the longest shoots were 15 and 12.7 mm respectively obtained at density of four explants and the shortest was 10.7 and 8.7 mm obtained at density of one explant per culture. At fixed volume of 3 ml per culture, the longest shoot was 15 mm obtained in cultures containing 2 and 3 explants and the shortest shoot was 8.7 mm obtained in cultures containing four explants. In cultures containing 5 ml the longest shoot was 15 mm obtained at density of two and the shortest was 13 mm obtained at three different explants density (1, 3 and 4 explants per culture). On the contrary, in cultures containing 6 ml, the longest shoot was 15 mm obtained at three different explants density (1, 3 and 4 explants per culture) and the shortest was 10.3 mm at density of two explants per culture (Table, 3.24). Similar, at fixed density of one explant per culture the longest shoot was 14.7 mm obtained

at two different volumes of medium (4 and 6 ml) and the shortest was 10 mm obtained in cultures containing 12 ml of medium. At density of two explants, the longest shoot was 19.7 mm obtained in cultures containing 7 ml and the shortest was 10 mm obtained at two different volumes of medium (10 and 11 ml). At density of three explants, the longest shoot was 16.7 mm obtained in cultures containing 12 ml of medium and the shortest was 12 mm obtained in the other volumes. At density of four explants, the longest shoot was 15 mm obtained in cultures containing 8 ml and the shortest was 8.7 mm obtained in two different volumses of medium (3 ml and 11 ml). That is, using of improper medium volume per culture caused loss of about 38 % of the shoot length and using of improper explants density caused loss of about 28 % of the possibly obtainable shoot length.

Comparing the effect of explants density on shoot length at each medium volume with the shoot length average overall medium volumes indicated that the explants density effect in shoot elongation followed four different patterns. The shoot length at 8, 9 and 10 ml of medium (Figure, 3.15A) increased as explants density increased up to 4 explants per culture. The shoot length at 3 and 12 ml (Figure, 3.15B) and 4 and 11 ml (Figure, 3.15C) increased as explants density increased to three explants and decreased afterward. However, while the increase in shoot length in cultures containing 3 and 12 ml of medium was sharp and in both volumes was above the average overall medium volumes, the shoot length increase in cultures containing 4 and 11 ml was gradual and that of 11 ml was below and that of 4 ml was above the shoot length average overall medium volumes. Dispensing the medium at 5, 6 and 7 ml per culture resulted in different shoot length curve. Except at density of two, the shoot length at 6 ml was above and at 5 and 7 ml of medium was below the shoot length average overall medium volumes.

While combinations of large volume of medium (above 9 ml) and low density (one explant) per culture resulted in higher shoot formation per explant (Table, 3.24), the

opposite, small volume of medium (3 and 4 ml) and high density of explants (three per culture) produced the highest total shoots per liter of medium (Table, 3.25). Dispensing the medium at any volumes except 7 and 10 ml per culture, using of one explant per culture resulted in the lowest total shoots per liter and at any volume except 8 and 10 ml per culture, using of three explants resulted in the highest total shoots per liter (Table, 3.25). At 10 ml, density of two explants per culture resulted in the lowest total (1000 shoots) and density of four resulted in highest total (2800 shoots) per liter. Dispensing the medium at 7 ml, the lowest total obtained at density of one, two and four explants per culture and the highest total shoots at density of three explants (2143 shoots). Dispensing the medium at 8 ml, density of one explant resulted in lowest total (1000 shoots) but the highest total (2500 shoots) obtained at density of four instead of density of three explants. At fixed explants density of one, two and four explants per culture, dispensing one liter of medium at 3 ml per culture resulted in the highest total of 3000, 4667 and 5333 shoots per liter while dispensing the medium at11, 10 and 12 ml per culture resulted in the lowest total of 727, 1000 and 1333 shoots per liter respectively. On the other hand, at density of three explants, one extra ml is needed (4 ml / culture) to obtain the highest total of 7500 shoots per liter and dispensing the medium at 7 ml per culture resulted lowest shoot total (2143 shoots). The highest total shoot per liter of all treatments were 7500 shoots obtained at density of three explants and 4 ml of medium per culture and the lowest were 727 shoots obtained at density of one explant and 11 ml of medium per culture.

The cost per shoot ranged from 3 to 15 cents (Table, 3.25). Increasing the explant density per culture decreased the cost per shoots from 11 cents at density of one explant to 4 cents at density of three explants. Increasing the medium volume per culture had little effect on the cost per shoot but decreased the total cost substantially. While the cost per one liter of medium dispensed at 3 ml per culture was RM 304.97, the cost of medium

dispensed at 12 ml per culture was only RM 86.68 but resulted in lower total shoots (Table, 3.25). Cost as low as 3 cents per shoot was obtained at several combinations of medium volumes and explants density, but at different total of cost and total of shoots. None of the medium volumes at density of one explant resulted in shoot cost less than 8 cents per shoot while all volumes and density of three explants per culture, the cost per shoot was less than 5 cents. At density of two explants, there was only one volume (11 ml) and at density of four explants, four of the medium volumes (4, 8, 9 and 10 ml) resulted in cost less than 5 cents. Over 50 % (2.5 cents per shoot) of the shoot cost was due to electricity bill of the incubation room. About 0.9 cents was due to labor wages and 0.8 cents for the culture tube (Figure, 3.16).

Shoot formation rate per explant could be used to screen the effectiveness of different treatments and indicates potential but without proper management, the rate alone could not be used as indicator for mass production of low cost propagating materials. The highest shoot formation per explant (13 shoots) obtained at 10 ml and density of one explant per culture (Table, 3.24). However, the cost per shoot was three times higher (8.0 cents) and total shoots (1300) were 6 times less (Table, 3.25) than that obtained in the combination of 4 explants and 4 ml per culture (7500 shoots and 3.0 cents per shoot). The second best total per liter (6000 shoots) obtained when three explants and 3 ml of medium were used per culture but the shoot cost was two times higher (6.0 cents per shoot). Keeping the same explants density (three explants per culture) and increasing the medium to 6 ml per culture decreased the total shoots per liter to 5000 shoots but reduced the cost per shoot by 50 % (6.0 to 3.0 cents).

Cost is the main obstacle of commercial micropropagation of pineapple. However, the production cost could not be minimized unless the effect of various factors of multiplication are assessed in association with their effect on the cost of medium, operating

time of autoclave, laminar and incubation room and shelving space, labor working hours and amount of containers. Investigation the effect of different explants density and medium volume per culture and assessment of the effect using total shoot per liter, cost per shoot and total cost are best save guard against any wasteful use of medium and shelving space and it is a proper parameter for comparison the cost of different protocols and a suitable approach to lower the cost of production. It also provide the propagators with several alternatives for production of low cost shoot depending on the budget and time and amount of shoots to be transfer to the next cycle of multiplication or rooting stage. A different combination of explants density and medium volume per culture may suit small company with limited budget and limited market access and other combination for big company with huge budget and world wide market access. Different total shoot per liter could be produced at equal cost by dispensing the medium at different volume per culture. Using of three explants and dispensing one liter of medium at 4, 6, 9 and 12 ml per culture resulted in total of 7500, 5000, 3667 and 2750 shoots per liter of medium all at cost of 3 cent cost per shoots but required investment of different amount of money RM 232.06, RM 159.14, RM 111.14 and RM 86.68 respectively (Table, 3.25).

Escalona, *et al*. (1999) recommended bioreactor system to increase multiplication rate and save shelving space and expected a cost reduction by more than 30%. Fifty clusters of three shoots (150 shoots) were cultured in a 10-liters bioreactor (10 liter of medium) for 7 weeks to produce a total of 2412 shoots. According to the result of our study, 150 shoots could be simply cultured at density of three explants and 4 ml per culture using only a total of 200 ml and 50 culture tubes, instead of the 10 liter bioreactor, and produce a total of 1500 shoots in 75 days. Just in term of medium, the cost is 30 times less than that of the temporary immersion system. Be and Debergh (2006) suggested outdoor incubation instead of artificially lightened incubation room. Figure, 3.16 shows that over 50 % of the

shoot cost was due to electricity bill of the incubation room. Adoption of outdoor incubation would reduce the electricity cost of incubation. Reusing of the culture tubes would save 16 % of the shoot cost, however, it will increase the laminar operating time and labor working hours. If the labor wages and electricity fare are not too high, reusing of the culture tubes could be possible means of lowering the cost of shoots during multiplication stage.

The *in vitro* shoot formation of Moris pineapple was significantly affected by the explants density and the volume of MS medium per culture. The shoot formation capacity decreased but the shoot elongation and estimated total per liter of medium increased as the explants density per culture increased. At different explant density there were different optimal of medium volume per culture for shoot formation, shoot elongation per explant and total shoot per liter. However, neither the effect of the explants density nor the medium volume per culture on the shoot formation capacity is well understood. Generally, using of more than one explants per culture suppressed the shoot formation capacity. At 5 and 8 ml of medium per culture, every added explant had almost equal suppression effect and the loss of proliferation ability proceeded at steady rate. However, in cultures containing 3 and 7 ml of the medium, adding of the second explant caused drastic loss on the shoot formation ability of the two explants while adding extra one (three explants) and two (four explants) caused a limited loss compared to density of two explants (Figure, 3.14B). On contrary, other medium volumes reversed the inhibition effect of explants density. At 4 and 12 ml of the medium, the shoot formation capacity increased by increasing the density up to three and at 11 ml, the proliferation improved by placing two rather than one explant in one culture (Figure, 3.14A).

Using bioreactor system, Escalona *et al.* (1999) reported that the optimal medium volume for pineapple proliferation was 200 ml per each cluster of three shoots. Chu *et al*.

(1993) reported that rose shoot cultured in 30, 40 and 50 ml of medium produced more shoots than those cultured in 10 ml while 20 ml of medium resulted in an intermediate effect. For Japanese yam 40 ml of medium per culture resulted in better shoot rate and fresh weight than 20 ml (Kadota and Niimi, 2004). At different volumes of 30, 50, 70 and 100 ml, the best multiplication of *Gladiolus* obtained in 50 ml and decreased afterwards. For tea nodal culture, Sandal *et al.* (2001) reported that 20 ml was the best, followed by 10 and the lowest rate obtained at 50 ml. Chun *et al*. (1986) found that including of more than two explants decreased the shoot formation of *Populus*. However, the number of shoots per vessel at density of 2 and 3 explants was twice as that at density of one explant. Different volumes of medium have different amount of nutrients and hormones and nutrients depletion is supposed to be the major cause of any differences in shoot formation. But, neither the results of this study, nor the literature is so supportive. This experiment was designed so that the different volumes and equal density reflect the important of nutrient supply while equal volume but different densities reflect competition between explants. Reversing of the inhibition effect of explant density on shoot formation in cultures containing 3, 5, 7 and 8 ml (Figure, 3.14B) by increasing the volumes to 11 and 12 ml (Figure, 3.14A) suggested a competition as a possible explanation of the medium volumes and explants density effect. However, the shoot formation in cultures containing 4 and 12 ml (Figure, 3.14A) and 6, 9 and 10 ml of medium (Figure, 3.14C) did not support that the effect of explant density and medium volume on the shoot formation capacity was related to nutrient supply and competition among explants. It is not clear how two extremes of volume (4 and 12 ml) could have almost the same effect on the shoot formation process.

Cousson *et al.* (1988) reported that the morphogenic mode of tobacco thin cell culture is a function of significant interaction between explants density, pH and volume of medium.

Toyoki *et al*. (1995) reported that in culture vessel containing 16 ml of medium some element depleted faster than in culture containing 32 ml of medium. In double state of solid and liquid medium, Han *et al.* (2004) found that the effectiveness of liquid addition in the induction of bullets formation of *Lilium longiflorum* depended on its sucrose content and the volume of the solid medium. Nhut *et al*. (2002) reported that at equal volume of regeneration medium, the protocorme like body of *Lilium longiflorum* thin cell layer that were induced in optimum volume of induction medium regenerated more shoots than those induced in improper volume. That is the volume of medium during protocorm induction effect the commitment of the protocorm to regenerate shoots even that the volume of shoot regeneration medium were equal. Sarker *et al.* (1997) reported a significant effect of density on potato shoot formation at short incubation of 2 weeks but that effect disappeared if the incubation extended for another two weeks.

An explant density-controlled and volume-dependent release of chemicals and gases that could be diluted below the effective level for promotion and inhibition of shoot formation by the volume of medium per culture seemed reasonable explanation. It is well known that the explants adjust the pH of the medium to a certain value. However, the value and time of equilibrium varied depend on plant type and explants density and medium states. Escalona *et al.* (1999) reported that pineapple culture reached an equilibrium value of 3.5 after 6 weeks of incubation. Leifert *et al.* (1992) reported that the time required for *Hemerocallis* culture to adjust the medium pH decreased from 35 days at density of one explant to 10 and 4 days at density of 5 and 10 explants per culture. Adjustment of the pH may also depend on the medium volume per culture. Furthermore, presence of more than one explant in one culture may affect the capability of each other for performing of certain physiological processes. In fact, Konan *et al*. (2007) reported that at density of three shoots per culture, the rooting of oil palm shoots were under so called

Table (3.24). Effect of explants density and medium volumes per culture on the *in vitro* shoot formation and shoot length of Moris pineapple.

Shoots	**MS volume per culture (ml)**									
/culture	3	4	5	6	7	8	9	10	11	12
Shoot formation per explants										
1	11 ab	6 bcde	8 abcde	8 abcde	11 ab	8 abcde	11 ab	13 a	8 abcde	9 abcd
2	6 bcde	7 bcde	7 bcde	5 cde	5 cde	7 bcde	6 bcde	5 cde	10 abc	9 abcd
3	6 bcde	10 abc	6 bcde	10 abc	5 cde	6 bcde	11 ab	9 abcd	8 abcde	11 ab
4	4 de	5 cde	3 e	3 e	3 e	5 cde	6 bcde	7 bcde	4 de	4 de
Aver.	6.7 NS	7 NS	6 NS	6.5 NS	6 NS	6.5 NS	8.5 NS	8.5 NS	7.5 NS	8.2 NS
Shoot length (mm)										
1	10.7 cde	14.7 abcd	12.7 bcde	14.7 abcd	11 cde	10.7 cde	12.7 bcde	8.7 e	11 cde	10 de
2	14 7 abcd	15.3 abc	15 abcd	10.3 cde	19.7 a	11.7 bcde	13.3 bcde	10 de	11 cde	13.3 bcd
3	15 abcd	15 abcd	13 bcde	15 abcd	12 bcde	13.7 bcde	13.7 bcde	12.7 bcde	11.7 bcde	16.7 ab
4	8.7 e	11 bde	13.3 bcde	15 abcd	12.3 bcde	14.7 abcd	13.3 bcde	12.7 bcde	9.3 e	11.7 bcde
Aver	12.2 ABC	14 A	13.7 A	13.7 A	13.7 A	12.7 ABC	13.2AB	11 BC	10.7 C	12.9 ABC

Data were average of three culture tubes containing static liquid MS medium (sucrose at 20 g/ l and BAP at 2.0 mg/l and pH adjusted to 5.0).
Means followed by same letters were not significantly different as tested by Duncan Multiple Range Test at $p \leq 0.05$.

Table (3.25). Effect of explants density and medium volumes per culture on the total shoots per liter, total cost and cost per shoot of Moris pineapple.

Shoots	**MS volume per culture (ml)**									
/cult	3	4	5	6	7	8	9	10	11	12
Total shoots per liter										
1	3667 def	1500 hijk	1600 hijk	1333 hijk	1571 hijk	1000 jk	1222 ijk	1300 hijk	727 k	750 k
2	4000 cde	3500 efg	2800 efgh	1667 hijk	1429 hijk	1750 hijk	1333 hijk	1000 jk	1818 hijk	1500 hijk
3	6000 b	7500 a	3600 efg	5000 bcd	2143 ghijk	2250 fghijk	3667 def	2700 efghi	2182 fghijk	2750 efghi
4	5333 bc	5000 bcd	2400 fghij	2000 hijk	1714 hijk	2500 fghij	2519 fghij	2800 efgh	1455 hijk	1333 hijk
Aver	4750 A	4375 A	2600 B	2500 BC	1714 C	1875 CD	2185 BCD	1950 BCD	1545 C	1583 C
Total cost per liter (RM)										
	304.97	232.06	188.31	159.14	139.10	123.37	111.14	101.36	93.36	86.68
Cost per shoot (RM)										
1	0.08	0.15	0.12	0.12	0.09	0.12	0.09	0.08	0.13	0.12
2	0.08	0.07	0.07	0.10	0.10	0.07	0.08	0.10	0.05	0.06
3	0.05	0.03	0.05	0.03	0.06	0.05	0.03	0.04	0.04	0.03
4	0.06	0.05	0.08	0.08	0.08	0.05	0.04	0.04	0.06	0.07
Aver	0.07	0.07	0.08	0.08	0.08	0.07	0.06	0.06	0.07	0.07

Explants were culture at density of 3 shoots per culture in liquid full strength MS supplemented with sucrose at 20 g/l and BAP at 2.0 mg/l and incubated at constant temperature 25 ^{0} C and 16 hour of light for 60 days.
Cost calculation were limited to variable cost items (Culture tubes, MS, sucrose, hormone, electricity for operating laminar, autoclave and incubation room, labor wages)
Means followed by same letter were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range test.
RM Malaysia Ringgit (1 USA $ = RM 3.25).

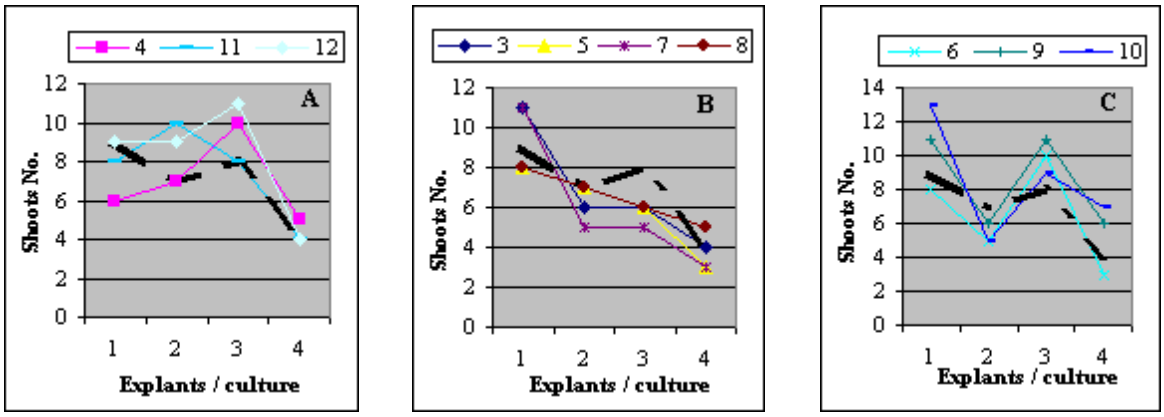


Figure (3.14). Effect of medium volumes A (4, 11 and 12 ml), B (3, 5, 7 and 8 ml) and C (6, 9 and 10 ml) on the pattern of shoot formation of Moris pineapple cultured at different explants density in static liquid MS medium enriched with sucrose at 20 g/l and BAP at 2.0 mg/l for 75 days.
Dot lines are average effect of explants density over 10 different volumes of medium.

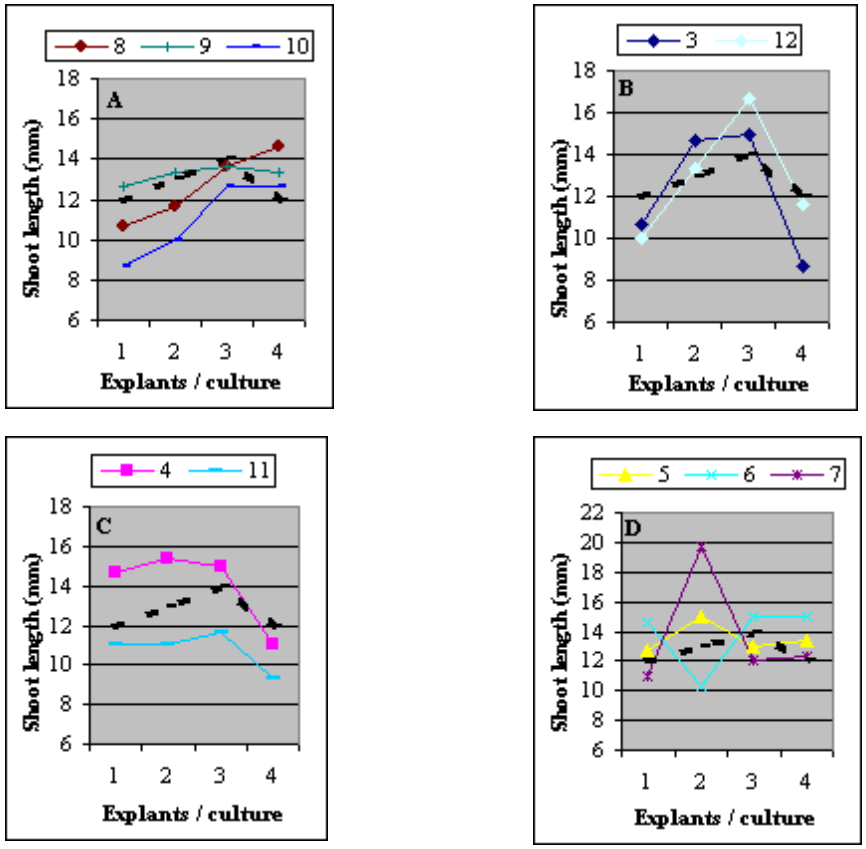


Figure (3.15).. Effect of medium volumes A (8, 9 and 10 ml), B (3 and 12 ml), C (4 and 11 ml) and D (5, 6 and 7 ml) on the pattern of shoot length of Moris pineapple cultured at different explants density in static liquid MS medium enriched with sucrose at 20 g/l and BAP at 2.0 mg/l for 75 days.
Dot lines are the average effect of explants density over 10 different volumes of medium.

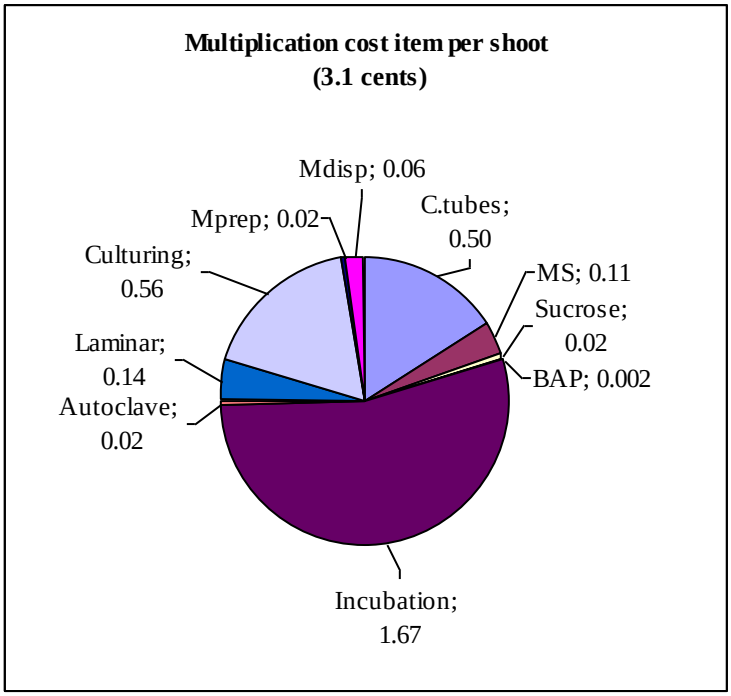


Fig. 3.16. Multiplication cost items per shoot of Moris. Explants were cultured at density of three explants per culture containing 4 ml of full strength liquid MS medium enriched with sucrose at 20 g/l and BAP at 2.0 mg/l and incubated under constant temperature 25 ^{0} C and 16 hours of light for 75 days.
Mprep. = (Medium preparation); Mdisp. = (Medium dispensing), Ctube.= (Culture tubes).
Culturing (separation and culturing of shoots under laminar)
Laminar, Autoclave, Incubation (electricity cost for operating laminar, autoclave and incubation room).

Plate (3.5) Shoot formation per explant of Moris pineapple in 6 ml of liquid full strength MS enriched with sucrose at 20 g/l, BAP at 2.0 and pH 5.0 at density of one (above) and density of three explants per culture (below) after 75 days of incubation.

coupling effect between the shoots of different size. Similar coupling effect may also exist between shoots during multiplication and one shoot effect on the proliferation of the others. Besides the size of the shoots, the coupling effect may also relate to the volume of medium per culture.

3.2.12. Effect of incubation periods, medium volumes and explants density on the *in vitro* proliferation and growth of Moris pineapple

The highest shoots formation per explant was 11 shoots obtained in 5 combinations of explants density, medium volumes per culture and incubation periods (Table, 3.26). In each of these 5 combinations the incubation period was longer than 45 days and none included density of two explants per culture. Only in one of the five combinations the incubation period was 60 days at density of three explants while the other four combinations the incubation period was 75 days at density of one and three explants and the medium volumes varied from 3 to 12 ml per culture. The lowest shoots formation, on the other hand, was 3 shoots per explant obtained in 6 combinations. In all of these combinations, the incubation was shorter than 60 days, the medium less than 9 ml and the explants density was more than one explant per culture. Generally, at all explants density and medium volumes per culture, increasing the incubation period increased the shoots formation per explant. However, the effect of explants density and medium volumes per culture on the shoots formation depended on the combination of the three factors, incubation periods, explants density and medium volumes per culture.

Compared to the 30 days incubation, at any explants density and medium volumes per culture, increasing the incubation period to 75 days increased the shoot formation per explant (Table, 3.26). However, whether incubation for 45 and 60 days produced more shoot than that obtained after 30 days incubation or less shoots than those obtained after 75

days depended on the combination of explants density and medium volumes. At fixed density of one explants in cultures containing 3 ml of medium each 15 days increase in the incubation (30, 45, 60 and 75 days) increased the shoot formation per explant (4, 8, 9 and 11 shoots). In cultures containing 6 ml of medium, incubation for 45 day resulted in more shoots per explant (6 shoots) than incubation for 30 days (4 shoots) but equal to that obtained after 60 days and extending the incubation to 75 days doubled the shoots formation (8 shoots) compared to 30 days incubation. In cultures containing 9 ml of medium, incubation for 30, 45 and 60 days resulted in equal shoots formation per explant (8 shoots) while incubation for 75 days increased the shoots formation to 11 shoots per explant. In cultures containing 12 ml of medium, increasing the incubation from 30 to 45 and 60 days increased the shoots formation from 5 to 6 and 9 shoots per explant. Extending the incubation to 75 days did not increase the shoots formation compared to that obtained after 60 days (9 shoots).

Similar, at fixed density of two explants and volume of 3 ml per culture incubation for 30 and 45 days resulted in equal shoots formation per explant (3 shoots) but increasing the incubation to 60 and 75 days increased the shoots formation to 4 and 6 shoots per explant respectively. At fixed volume of 6 ml, incubation for 30, 45 and 60 days resulted in equal shoots formation per explant (4 shoots) but increasing the incubation to 75 days increased the shoots formation to 5 shoots per explant. At fixed volume of 9 ml, increasing the incubation from 30 to 45 and 60 days increased the shoots formation from 3 to 5 and 6 shoots per explant. Extending the incubation to 75 days did not increase the shoots formation compared to that obtained after 60 days (6 shoots). At fixed volume of 12 ml, incubation for 45 day resulted in more shoots per explant (5 shoots) than incubation for 30 days (3 shoots) but equal to that obtained after 60 days and extending the incubation to 75 days tripled the shoot formation (9 shoots) compared to 30 days incubation. On the other

hand, at fixed density of three explants and 6 and 9 ml per culture each 15 days increase in the incubation (30, 45, 60 and 75 days) increased the shoot formation per explant (3, 4, 6 and 10 shoots) and (5, 6, 7 and 11 shoots) respectively while at fixed volume of 3 ml, incubation for 30 and 45 days resulted in equal shoots formation per explant (3 shoots) but increasing the incubation to 60 and 75 days increased the shoots formation to 4 and 6 shoots per explant respectively. At fixed volume of 12 ml, increasing the incubation from 30 to 45 and 60 days increased the shoots formation from 5 to 7 and 11 shoots per explant. Extending the incubation to 75 days did not increase the shoots formation compared to that obtained after 60 days (11 shoots). In other word, the proper incubation period depended on the volume of medium dispensed per culture. At certain volume of medium increasing the incubation was just unjustifiable extra electicity cost.

The effects of explants density per culture on the shoots formation per explant were also depended on the medium volumes per culture and incubation periods. In all combinations of incubation periods and medium volumes per culture, density of two explants resulted in the lowest rate of shoot formation per explant. However, whether explants density of one resulted in more or less shoots formation than density of three explants depended on the combination of incubation periods and medium volume per culture. Increasing the explants density from one to two explants per culture decreased the rate of shoot formation per explant at all medium volumes per culture and all incubation periods except at one combination. Equal shoot formation (4 shoots per explant) obtained at density one and two explants incubated for 30 days in 6 ml of medium. Increasing the explants density from one to three explants per culture decreased the rate of shoot formation per explant at the combinations in which the medium volumes per culture were less than 12 ml and incubation periods shorter than 75 days. On the contrary, in cultures containing 12 ml, at all incubation periods and cultures incubated for 75 at all medium

volumes, increasing the explants density from one to three explants per culture increased the shoot formation per explant (Table, 3.26).

At each incubation period (30, 45, 60 and 75 days) and 3 ml of medium per culture, density of one explant per culture resulted in higher shoot formation (4, 8, 9 and 11 shoots) while two and three explant per culture resulted in lower shoots formation per explant (3, 3, 4 and 6 shoots). At 6 ml of medium per culture, one explant per culture resulted in higher shoots formation than three explants after 30 days (4 and 3 shoots respectively) and 45 days (6 and 4 shoots respectively) of incubation. On the contrary, after 60 days equal shoots formation on both explants density (6 shoots) and after 75 days, density of three explants resulted in higher shoots formation than density one explant. At 9 ml of medium, one explant per culture resulted in formation of 8 shoots per explant after 30, 45 and 60 days of incubation while three explants per culture resulted in formation of fewer shoot per explant (5, 6 and 7 shoots per explant respectively). However, after 75 days of incubation, both explants density resulted in equal shoot formation (11 shoots). At 12 ml, equal shoot formation (5 shoots) obtained at density of one and three explants per culture after 30 days of incubation while after 45, 60 and 75 days of incubation, the three explants per culture resulted in higher shoot formation (7, 11 and 11 shoots) than density of one explant (6, 7 and 9 shoots). Increasing the explants density from two to three explants per cultures increased the shoots formation per explant in cultures containing 6, 9 and 12 ml at all incubation periods (Table, 3.26). However, in cultures containing 3 ml of medium, using of two and three explants per culture resulted in equal shoot formation per explant (3, 3, 4 and 6 shoots) at 30, 45, 60 and 75 days of incubation respectively.

The effect of the medium volume per culture on shoot formation per explant varied at different explants density and incubation periods (Table, 3.26). At density of one explant per culture, cultures containing 3 ml produced more shoots per explant than cultures

containing 12 ml of medium after 45, 60 and 75 days incubation. Cultures containing 3 ml produced also more shoots (9 shoots) than cultures containing 9 ml (8 shoots) at 60 days of incubation. However, both 3 and 9 ml of medium resulted in equal rate after 45 (8 shoots) and 75 days of incubation (11 shoots). Both of 6 and 12 ml of medium resulted in equal shoot formation (6 shoots) after 45 days of incubation. However, after 60 and 75 days, cultures containing 12 ml produced more shoots (7 and 9 shoots) than that containing 6 ml of medium (6 and 8 shoots). On the contrary, after 30 days incubation cultures containing 12 ml produced more shoots (5 shoots) than cultures containing 3 and 6 ml (4 shoots) but less than that in culture containing 9 ml (8 shoots). On the other hand, at density of two explants per culture and incubation of 30 days, equal shoot formation (3 shoots each) obtained at different medium volumes per culture. However, while after 45 days incubation cultures containing 9 and 12 ml of medium resulted in equal shoot formation (5 shoots), 9 ml resulted in more shoots (6 shoots) than 12 ml of medium (5 shoots) if the incubation extended to 60 days and 12 ml resulted in more shoots (9 shoots) than 9 ml of medium (6 shoots) if the incubation extended to 75 days. After 45 days, cultures containing 6 ml produced more than that containing 3 ml while after 60 days equal shoots formation obtained at both medium volumes. On the contrary, after 75 days, 3 ml produced more shoots than 6 ml. The same trend of interaction effect of medium volume per culture and incubation periods was also observed using density of three explants per culture. The shoot formation at 45 days increased from 3 to 4, 6 and 7 shoots as the volume per culture increased from 3 to 6, 9 and 12 ml per culture and the shoot formation at 60 days increased from 4 to 6, 7 and 11 shoots as the volume per culture increased from 3 to 6, 9 and 12 ml per culture respectively. In these two incubation periods (45 and 60 days) every three ml increase in volume resulted in more shoot formation per explant. However, the trend was different if the explants incubated for 30 and 75 days. In cultures incubated for 30 days, the

shoot formation increased from 3 to 5 shoots as medium volume increased from 6 to 9 ml per culture and in cultures incubated for 75 days the shoot formation increased from 6 to 10 shoots as the medium volume increased from 3 to 6 ml per culture. However, dispensing the medium at 9 and 12 ml per culture resulted in equal shoot formation after 30 days (5 shoots) and 75 days of incubation (11 shoots). Although, the different in shoot length between the various combinations of medium volume, explants density per culture and incubation periods were statistically significant and some combinations doubled the length compared to others (Table, 3.26), in practice the shoot length were almost equal. The maximum different between the shortest and the longest shoot was less than one cm. The shoot length increased as the incubation periods increased particularly when three explants were used per culture. Increasing the medium volume per culture decreased the shoot length if the explants incubated for 30 and 45 days but had no effect on shoot length if the explants incubated for 60 and 75 days.

The highest total of shoots per liter (6000 shoots) obtained when the medium dispensed at 3 ml and three explants placed per culture and incubated for 75 days and the lowest total (417 shoots) obtained when the medium dispensed at 12 ml and one explant placed per culture and incubated for 30 days (Table, 3.27). Generally, higher density, smaller volume and longer incubation resulted in highest total of shoot per liter. However, equal total of shoots per liter could be obtained at different incubation periods at proper combination of explants density and medium volumes per culture and vice versa. At density of one explant and 3 ml of medium per culture and at density of three explants and 6 and 9 ml of medium per culture every 15 days increase in incubation period resulted in increase in total shoots per liter (longer incubation resulted in higher total than shorter incubation). However, equal total was obtained at 45 and 60 days of incubation (1000 shoot each) using density of one explant and 6 ml of medium per culture. Both totals were higher than that after 30 days

(667 shoots) and increasing the incubation to 75 days increased the total to 1333 shoots. At density of one explant and 9 ml of medium per culture, equal total (889 shoots) obtained after 30, 45 and 60 days and increasing the incubation to 75 days increased the total to 1222 shoots per liter. At 12 ml of medium equal total obtained at 45 and 60 days of incubation (about 542 shoot each), both totals was higher than that after 30 days (416.7 shoots) and increasing the incubation to 75 days increased the total to 750 shoots.

Similar, at density of two explants and 3 ml of medium per culture, equal total obtained after 30 and 45 days (2000 shoots) and increasing the incubation to 60 and 75 days increased the total to 2667 and 4000 shoots per liter. At 6 ml of medium, equal total obtained after 30, 45 and 60 days (1333 shoots) and increasing the incubation to 75 days increased the total to 1667 shoots. On contrary, at 9 ml of medium, incubation for 45 days resulted in more total (1111 shoots) than that at 30 days (667 shoots) but increasing the incubation to 60 and 75 days did not result in significant different in total (1333 shoots) than that obtained at 45 days of incubation. At 12 ml of medium, equal total of shoot (833 shoots) obtained at 45 and 60 days of incubation but more than that at 30 days (500 shoots) and increasing the incubation to 75 days increased the total to 2125 shoots. At density of three explants and 3 ml of medium, equal total of shoot (3000 shoots) obtained at incubation of 30 and 45 days and the total increased as the incubation increased to 60 (4000 shoots) and 75 days (6000 shoots). At 12 ml of medium, the total increased as the incubation increased to 60 days but the total at 75 days was equal to that at 60 days (2750 shoots each).

At all incubation periods and medium volumes per culture the lowest total shoot per liter of medium obtained at density of one explant, highest total at density of three explants and intermediate total at density of two explants per culture except in cultures containing 9 ml of medium and incubated for 30 days and cultures containing 3 ml of medium and

incubated for 45 and 60 days. At these combinations, density of two explants resulted in lowest and intermediate total obtained at density of one explant per culture (Table, 3.27). At all incubation periods dispensing the medium at 3 ml and using of three explants per culture resulted in highest total per liter and dispensing at 12 ml and using of one explant per culture resulted in lowest total shoots except at density of two explants and 75 days incubation and at density of three explants and 60 days incubation where the lowest total shoots (1333 and 2333 shoots) obtained in cultures containing 9 ml instead of 12 ml (Table, 3.27). At incubation of 30 days and all explants density and incubation of 75 days and density of three explants per culture each 3 ml increase in medium per culture decreased the total shoots per liter (Table, 3.27). However, at 45 days incubation and all explants density, 60 days incubation and explant density of one and two and 75 days incubation and explant density of one explant per culture, dispensing the medium at 3 ml per culture resulted in highest and dispensing at 12 ml per culture resulted in lowest total shoots per liter but equal total shoots per liter obtained in medium dispensing at 6 and 9 ml per culture (not significant different). Equal total shoots per liter could be obtained even if the medium was dispensed at different volumes per culture provided different combination of incubation periods and explants density were used. At fixed density of three explants and different incubation periods, dispensing the medium at 3 and 6 ml per culture resulted in equal total of 3000 shoots per liter. At fixed incubation of 45 days and different explants density dispensing the medium at 3, 6 and 9 ml per culture resulted in equal total of 2000 shoots per liter. At different combination of explants density and incubation periods dispensing the medium at 3, 6, 9 and 12 resulted in equal total of 1333 shoots per liter (Table, 3.27).

The total cost per liter of medium at any medium volume per culture increased as the incubation period increased and at any incubation period the total cost decreased as the

medium volume per culture increased while the explants density per culture had no effect on the total cost (Table, 3.27). But, each one of these factors (explant density, medium volumes and incubation periods) affected the total shoots per liter and cost per shoot. Equal cost per shoot could be obtained at different combinations of incubation periods, medium volume per culture, but at different total cost and total shoots per liter. The highest total cost per liter was RM 304.72 and the lowest was RM 61.54 obtained respectively after 75 days of incubation in medium dispensed at 3 ml per culture and after 30 days of incubation in medium dispensed at 12 ml per culture. The highest cost per shoot was RM 0.16 obtained after 30 days incubation of one explant in cultures containing 6 ml of medium. The lowest cost per shoot was RM 0.03 obtained when three explants were cultured in 12 ml of medium for 60 days and in 6, 9 and 12 ml of medium for 75 days..But the total shoots obtained from these combinations of medium volumes and incubation periods were 2750, 5000, 3667 and 2750 shoots per liter at a total cost were RM 78.14, RM 159.23, RM 111.01 and RM 86.44 respectively. Equal cost of RM 0.05 per shoot was also obtained at equal explants density of three explants per culture but at different volumes of medium per culture and different incubation periods (three explants in 9 and 12 ml of medium per culture for 30 days; 6 ml of medium for 60 days and 3 ml of medium for 75 days incubation). Selection of which combination is the best depends on available budget and facilities and amount of propagules and time of delivery. Figure (3.17) shows that the major factor of cost was the electricity of incubation room (53 %) followed by labor wages (21 %) and culture tubes (16 %).

In most of the previous *in vitro* multiplication studies of pineapple, the explants density, medium volume and the incubation periods were usually fixed at one explant, 20 ml of medium per culture and 30 days and the treatment assessed by comparing the shoot formation rate. Little wonder that the cost was the main obstacle of pineapple

micropropagation. Table 3.27 showed that irrespective of the medium volumes per culture and incubation periods, using of one explant resulted in higher cost per shoot than using of two and three explants. Similar, dispensing the medium at 12 ml per culture resulted in higher cost per shoot than other medium volumes irrespective of the incubation periods and explants density per culture and incubation for 30 days resulted in higher cost per shoot than other incubation periods irrespective of the medium volumes and density of explants per culture. In addition, using shoot formation rate indicated that five combinations resulted in the highest shoot formation per explant. But the difference in cost per shoot among these combinations was three times and the difference in total shoots was two times (Table, 3.27). In other word, if the cost was not analysed, treatment with high cost per shoot could be possibly picked up as the best treatment. The best combination of medium volume, explant density and incubation period for multiplication of Moris pineapple varied according to the parameter used for assessment. According to shoot formation rate five combinations (one explant in 3 and 9 ml of medium and three explants in 9 and 12 ml of medium all for 75 days incubation and three explants in 12 ml of medium for 60 days incubation) resulted in the highest shoot formation per explant (11 shoots). According to total shoots per liter, one combination (three explants in 3 ml of medium for 75 days incubation) resulted in the highest total (6000 shoots) and judging by cost per shoot four combinations (three explants in 6, 9 and 12 ml of medium for 75 days and three explants in 12 ml of medium for 60 days) resulted in the lowest cost per shoot (RM 0.03). None of the combinations was the best by all of the three parameters, shoot rate, total shoots and cost per shoot. Althoguh these combinations resulted in equal cost per shoot, they resulted in different total shoots (5000, 3667, 2750, and 2750 shoots) and different total cost (159, 111, 86 and 87 RM) per liter of medium respectively.

Although it is not only important for shoot formation but also as cost factor, the effect of medium volumes and explants density per culture on the shoot formation of pineapple in conventional system was not investigated before. Previous studies showed that at density of three explants and 10 ml of medium for 60 days (Hamad and Taha, 2003), two explants and 10 ml for 42 days (Danso *et al*., 2008), 30 ml for 60 (Be and Debergh, 2006), 20 ml for 70 days (Soneji *et al*., 2002a) and one explant and 50 ml for 42 days (Sripaoraya *et al*., 2003), 25 ml for 42 days (Aydieh *et al*., 2000), 20 ml for 75 days (Teixeira *et al*., 2006) and 30 days (Fitchet, 1990a) a rate of 6, 11, 9, 12, 10, 6, 13 and 9 shoots per explant were obtained respectively. The rate is comparable to our results taking in account that different hormone treatments and cultivar were used. This study indicated a medium volume as low as 3 ml per culture could be used for pineapple multiplication. In fact, at density of one explant, equal rate of shoot formation obtained at 3 and 9 ml of medium per culture and at density of three explants, dispensing the medium at 3 ml per culture resulted in the highest total shoots per liter. The three factors should be investigated simultaneously. The optimum level of one factor may vary depending on the combination of the other two factors. At density of one explant per culture and shorter incubation of 30 days, increasing the medium volume up to 9 ml per culture increased rate per explant. However, at longer incubation of 45, 60 and 75 increasing the medium volume per culture decreased the rate of shoot formation per explant. On the contrary, at density of three explants in all incubation periods and at density of two explants, except at shorter incubation of 30 days, increasing the medium volume per culture increased the shoot formation per explant.

The results of this study (Table, 3.27) showed that these factors (explant density, medium volume per culture and incubation periods) not only affected the shoot formation rate but also the total shoots and total cost per liter of medium as well as cost per shoot. The 3 ml of medium per culture used in this study is 7 times less than the most commonly

used medium volume (20 ml per culture) but the shoot formation (Table, 3.26) was about equal to those previously reported. The extra 17 ml of medium is just a possibly avoidable added cost. Similar, the cost per shoot (Table, 3.27) after 30 and 45 days of incubation which is the most commonly used incubation period for multiplication of pineapple, was 5 times higher (RM 0.16) than the cost after 75 days (RM 0.03). Arbitrary selection of incubations period, explants density and medium volumes per culture and assessment of treatments based on its effect on rate of shoot formation, which are usually done could not lead to obtaining the highest shoot production and the lowest cost per shoot. Without optimization of these three factors and including of all cost items in consideration, the cost of micropropagation could not be minimized and would always be a main obstacle of pineapple micropropagation. Naturally, dispensing the medium at smaller volume per culture increased the number of culture tubes and consequently the shelving space and the total cost would be increased. However, the cost per shoot depended on the effect of the medium volume and explant density per culture on the rate of shoot formation per explant. Volume which increased the rate would compensate for the higher cost of culture tubes and space and reduced the cost per shoot. At all incubation periods and explants density, dispensing the medium at smaller volume per culture resulted in higher total shoot and total cost per liter than dispensing the medium at larger volume per culture. However, while at density of three explants the cost per shoot decreased as the volume per culture increased, increasing the volume at density of one explant increased the cost per shoot (Table, 3.27). Incubations for 30, 45 and 60 resulted in equal total shoots per liter at density of one explant and 9 ml of medium per culture (889 shoots) and at density of two and 6 ml of medium (1333 shoots) but less than that after 75 days. That is both of 45 and 60 days were improper incubation period. They did not increase the total more than that at 30 days and both resulted in fewer total than that at 75 days. Either 30 or 75 days of

incubation could be used. Similar, incubation for 60 was not proper at density of one explant and 6 ml and density of two explants and 12 ml of medium but either, 30, 45 or 75 days could be used. At density of two explants and 9 ml of medium per culture equal shoot total per liter of medium obtained at 45, 60 and 75 days of incubation. That is, using of incubation longer than 45 days is just wasting of time and money. However, at 3 ml of medium and density of two and three explants per culture 45 days was also improper incubation. It did not increase the total more than that at 30 days and produced less totals than that at 60 and 75 days. At density of three explants and 12 ml of medium, 75 days was improper incubation period. Incubation longer than 60 days did not cause any significant increase in total shoot per liter of medium. The major cost factor during multiplication was the electricity cost of incubation period (Figure, 3.17). The cost of electricity varied at different countries one incubation period may recommend in one country but not for the others.

Cost per shoot is the best parameter for measuring the cost effectiveness of a protocol. However, the total production, on the other hand, is very crucial to meet certain obligation of market demand and time of delivery. None of the combination achieved these two goals (Table, 3. 27). The cost per shoot in the combination which resulted in the highest total per liter (6000 shoots) was two times (RM 0.05) higher and the total shoots in combination which resulted in the lowest cost per shoot (RM 0.03) was one fifth (5000 shoots) to two times less (2750 shoots). Since, the total production could be managed by increasing the amount of medium per single cycle of multiplication, the selection of best combination would be judged by the total cost and total shoot production per cycle. Three explants and 3 ml of medium and 75 days incubation resulted in 6000 shoot and cost of RM 304 per liter. The cost of using two liters of medium dispensed at 6 ml per culture was almost the same (RM 318) but would result in production of 10000 shoots. Dispensing two liters of

Table 3.26. Effect of incubation periods, medium volumes and explants density per culture on the in vitro shoot formation per explant and shoot length of Moris pineapple

MS	**Incubation periods (days) and explants density**											
(ml)	30			45			60			75		
	1	2	3	1	2	3	1	2	3	1	2	3
Shoots per explants												
3	4 e	3 e	3 e	8 bcde	3 e	3 e	9 bcd	4 e	4 e	11 b	6 cde	6 cde
6	4 e	4 e	3 e	6 cde	4 e	4 e	6 cde	4 e	6 cde	8 bcde	5 de	10 bc
9	8 bcde	3 e	5 de	8 bcde	5 de	6 cde	8 bcde	6 cde	7 bcde	11b	6 cde	11 b
12	5 de	3 e	5 de	6 cde	5 de	7 bcde	7 bcde	5 def	11 b	9 bcd	9 bcd	11 b
Shoot length (mm)												
3	8.7 bcd	11.7 abcd	9.3 bcd	9.7 bcd	11.7 abcd	12.3 abcd	10 bcd	13.7 abcd	11.7 abcd	10.7 abcd	14.7 abc	15 ab
6	8.3 cd	8.3 cd	9.3 bcd	10 bcd	9 bcd	8 d	11.3 abcd	9.7 bcd	12.7 abcd	14.7 abc	10.3 bcd	15 ab
9	7.3 d	7 d	7.3 d	7.7 d	7.7 d	8 d	9.3 bcd	12.3 abcd	12.3 abcd	12.7 abcd	13.3 abcd	13.7 abc
12	8 d	7.7 d	7 d	9 bcd	9 bcd	8.7 bcd	9 bcd	11.7 abcd	12 abcd	10 bcde	13.3 abcd	16.7 a

Explants (shoots) were cultured in culture tubes containing static liquid full strength MS medium enriched with sucrose at 20 g/ l and BAP at 2.0 mg/l and pH adjusted to 5.0 and incubated under constant temperature (25 ^{0} C) and 16 hours of light.
Means followed by same letters were not significantly different as tested by Duncan Multiple Range Test at $p \leq 0.05$.

Table (3. 27). Effect of incubation periods, medium volume and explants density per culture on the total shoots and total cost per liter of medium and cost per shoot of Moris pineapple

MS	Incubation periods (days) and explants density											
(ml)	30			45			60			75		
	1	2	3	1	2	3	1	2	3	1	2	3
Total shoots per liter												
3	1333 hij	2000 efghi	3000 cde	2667 defg	2000 efghi	3000 cde	3000 cde	2667 defg	4000 bc	3667 cd	4000 bc	6000a
6	667 j	1333 hij	1500 ghij	1000 ij	1333 hij	2000 efghi	1000 ij	1333 hij	3000 cde	1333hij	1667fghi	5000b
9	889 ij	667 j	1667 fghij	889 ij	1111 hij	2000 efghi	889 ij	1333 hij	2333efgh	1222hij	1333 hi	3666cd
12	417 j	500 j	1250 hij	500 j	833 ij	1750 fghij	583 j	833 ij	2750 def	750 j	1500ghi	2750def
Total cost per liter (RM)												
3	209.89	209.89	209.89	243.19	243.19	243.19	276.49	276.49	276.49	309.79	309.79	309.79
6	114.62	114.62	114.62	131.32	131.32	131.32	148.02	148.02	148.02	164.72	164.72	164.72
9	84.01	84.01	84.01	95.11	95.11	95.11	106.21	106.21	106.21	117.31	117.31	117.31
12	67.39	67.39	67.39	75.69	75.69	75.69	83.99	83.99	83.99	92.29	92.29	92.29
Cost per shoot (RM)												
3	0.16	0.10	0.07	0.09	0.12	0.08	0.09	0.10	0.07	0.08	0.08	0.05
6	0.17	0.09	0.08	0.13	0.10	0.07	0.15	0.11	0.05	0.12	0.10	0.03
9	0.09	0.13	0.05	0.11	0.09	0.05	0.12	0.08	0.05	0.10	0.09	0.03
12	0.16	0.13	0.05	0.15	0.09	0.04	0.14	0.10	0.03	0.12	0.06	0.03

Explants (shoots) were cultured in culture tubes containing static liquid full strength MS medium enriched with sucrose at 20 g/ l and BAP at 2.0 mg/l and pH adjusted to 5.0 and incubated under constant temperature (25^{0} C) and 16 hours of light.
Means followed by same letters were not significantly different as tested by Duncan Multiple Range Test at $p \leq 0.05$.
RM.Ringgit Malaysia (1 USA $= RM 3.25).
Cost estimate included only the variable cost items.

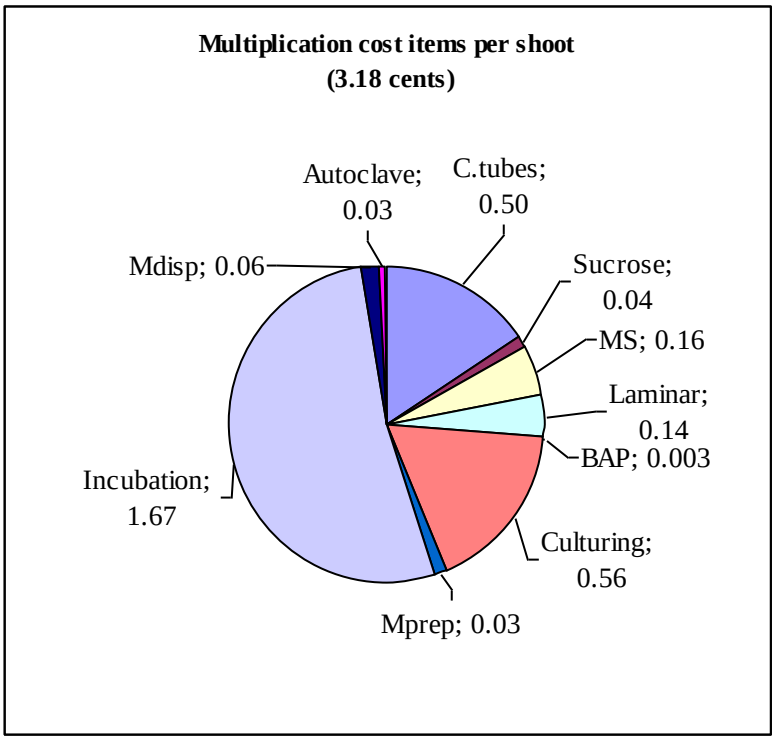


Figure (3.17). Multiplication cost items per shoot of Motis pineapple cultured at density of 3 explants in culture tube containing 6 ml of full strength liquid MS medium enriched with sucrose at 20 g/l and BAP at 2.0 mg/l. The cultures incubated under 16 hours of light and constant temperature 25^{0} C for 75 days**.**
Mprep. = (Medium preparation); Mdisp. = (Medium dispensing), Ctube.= (Culture tubes).
Culturing (separation and culturing of shoots under laminar)
Laminar, Autoclave, Incubation (electricity cost for operating laminar, autoclave and incubation room).

medium at 9 ml and incubation for 75 days would result in total of 7334 shoots and cost RM 222 and dispensing at 12 ml per culture and incubating for 60 days would produce total of 5500 shoots and cost RM 156. In all of these alternatives, the cost per shoot would be RM 0.03 and the choice would depend on budget and facility available and amount and time of propagules delivery.

3.2.13. Effect of strength, volume and pH adjustments of MS medium on proliferation and growth of Moris pineapple

Medium strength, pH and volume of medium dispensed per culture effected the *in vitro* shoot formation of Moris pineapple (Table, 3.28). The optimum combinations of each two of these three factors for shoot formation varied at different levels of the other one. At fixed medium strength of 1.0x, the highest shoot formation (8 shoots) obtained in medium adjusted to pH 5.0 and dispensed at 6 ml and in medium adjusted to pH 6.5 and dispensed at 8 ml per culture and the lowest (2 shoots) in medium adjusted to pH 5.5 and dispensed at 4 ml per culture. In medium adjusted to pH 5.0 and pH 6.5 increasing the medium volume per culture from 3 to 6 ml increased the shoot formation from 4 to 8 shoots. That is a different of 3 to 5 ml in medium volume per culture resulted in difference of 4 to 5 shoots per explant. At all medium volumes per culture except 8 ml, the best shoot formation obtained in medium adjusted to pH 5.0. However, while adjusting to pH 6.5 double the shoot formation (6 and 8 shoots) compared to pH 5.5 (3 and 4 shoots) in cultures containing 6 and 8 ml, equal shoot formation obtained in both pH adjustments (3, 3 and 4 shoots) in cultures containing 3, 5 and 7 ml.

At fixed medium strength 1.5x, the highest shoot formation (9 shoots) obtained in media adjusted to pH 5.0 and 5.5 dispensed at 7 ml per culture and the lowest shoot formation (3 shoots) in media adjusted to pH 5.5 and 6.5 and dispensed at 3 ml per culture.

In media adjusted to pH 5.0 and 5.5, increasing the medium volume per culture from 3 to 7 ml increased the shoot formation from 6 and 3 to 9 shoots while in medium adjusted to pH 6.5 increasing the medium volume per culture from 3 to 5 ml increased the shoot formation from 3 to 7 shoots. That is a different of 2 to 4 ml in medium volume per culture resulted in a different of 3 to 6 shoots on shoot formation per explant. At all medium volumes per culture except 5 ml, adjusting the medium to pH 5.0 resulted in higher shoot formation than other pH adjustments. On the contrary, adjusting the medium pH to 6.5 resulted in more shoots (7 shoots) than adjusting to pH 5.0 (6 shoots) and pH 5.5 (5 shoots) if the medium was dispensed at 5 ml per culture and in more shoots (5 shoots) than pH 5.5 (4 shoots) if the medium dispensed at 4 ml while adjusting the medium to pH 5.5 resulted in more shoots (9 shoots) than adjusting to pH 6.5 if the medium dispensed at 7 ml per culture (6 shoots). No different in shoot formation between medium adjusted to pH 5.5 and 6.5 and dispensed at 3, 6 and 8 ml (3, 5 and 6 shoots each) and between medium adjusted to pH 5.0 and 5.5 and dispensed at 7 ml per culture (9 shoots each).

In medium strength 2.0x, the highest shoot formation (10 shoots) obtained in medium adjusted to pH 5.0 and dispensed at 7 ml and the lowest (2 shoots) in medium adjusted to pH 6.5 and dispensed at 8 ml per culture. In medium adjusted to pH 5.0 and pH 5.5, increasing the medium volume per culture from 3 to 8 ml increased the shoot formation from 6 to 9 shoots but on contrary decreased the shoot formation in medium adjusted to pH 6.5 from 4 to 2 shoots. That is a 5 ml different in medium volume per culture could increase shoot formation per explant by 3 shoots and decrease the shoot formation by 2 shoots. At all medium volumes per culture the best shoot formation obtained in medium adjusted to pH 5.0. However, while in cultures containing 4, 5 and 7 ml each 0.5 increase in pH decreased the shoot formation per explant, no different in shoot formation between

medium adjusted to pH 5.5 and 6.5 and dispensed at 6 ml (5 shoots) and medium adjusted to pH 5.0 and 5.5 and dispensed at 3 (6 shoots) and 8 ml (9 shoots) per culture.

Fixing the media pH at 5.0, the highest shoot formation (10 shoots) obtained in medium strength 2.0x dispensed at 7 ml per culture and the lowest (1 shoot) in medium strength 0.5x dispensed at 7 ml. Medium strength 2.0x was the best strength at all medium volumes, except at 3 and 6 ml per culture. Equal shoot formation (6 shoots) obtained in medium strength 1.5x and 2.0x if the media dispensed at 3 ml per culture and medium strength 1.0x produced more shoots (8 shoots) than medium strength 1.5x (7 shoots) but equal to strength 2.0x if the media dispensed at 6 ml. In medium dispensed at 5 ml equal shoots formation (6 shoots) obtained in medium strength 1.0x and 1.5x but less than that on medium strength 2.0x (7 shoots). Similar, fixing the media pH at 5.5, the highest shoot formation (9 shoots) obtained in media strength 1.5x dispensed at 7 ml and medium strength 2.0x dispensed at 8 ml per culture and the lowest (2 shoots) in medium strength 0.5x dispensed at 3 and 4 ml per culture. The best strength for media dispensed at 3, 4, 5 ml was 2.0x resulted in formation of 6 shoots per explant. For media dispensed at 6 and 7 ml, the best strength was 1.5x resulted in formation of 5 and 9 shoots respectively. However, while increasing the strength to 2.0x did not effect the shoot formation of cultures containing 6 ml (5 shoots) it decreased the shoot formation of cultures containing 7 ml from 9 to 6 shoots. Fixing the medium pH at 6.5, the highest shoot formation (8 shoots) obtained in medium strength 1.0x dispensed at 8 ml per culture and the lowest (1 shoot) in medium strength 0.5x dispensed at 4 ml per culture. In medium dispensed at 5 and 7 ml, the best medium strength was 1.5 x resulted in formation of 7 and 6 shoots respectively while in media dispensed at 6 and 8 ml per culture the best strength was 1.0x resulted in formation of 6 and 8 shoots. The shoot formation in cultures containing 6 and 8 ml increased as the medium strength increased up to 1.0x while in cultures containing 4, 5

and 7 ml the shoot formation increased as the medium strength increased up to 1.5x and decreased afterwards.

Generally, except at medium strength 0.5x, dispensing the medium at higher volume per culture resulted in more shoot formation than dispensing at lower volume per culture. However, the proper medium volume per culture varied according to medium strength and pH adjustments. Media of different strengths could result in equal shoot formation depending on the pH adjustment and volume of medium dispensed per culture. In media dispensed at 4, 7 and 8 ml per culture the optimal strength was 2.0x adjusted to pH 5.0. Dispensing the media at fixed volume of 3 ml per culture, the highest shoot formation of all combinations of media strength and pH adjustments (6 shoots) obtained in medium strength 2.0x adjusted to pH 5.0 and 6.5 and in medium strength 1.5x adjusted to pH5.0. At fixed volume of 5 ml per culture, the highest shoot formation (7 shoots) obtained in medium strength 2.0x adjusted to pH 5.0 and medium strength 1.5x adjusted to pH 6.5 while at fixed volume of 6 ml the highest shoot formation (8 shoots) obtained in medium strength 2.0x and 1.0x both adjusted to pH 5.0.

The shoot length ranged from shortest (8 mm) obtained in combination of double strength MS medium (2.0x) adjusted to pH 6.5 and dispensed at 8 ml per culture to longest shoot (29 mm) obtained in combination of half strength MS (0.5x) adjusted to pH 5.0 and dispensed at 6 ml per culture. However, keeping one of these factors fixed the optimal combinations of the other two varied at different values of the fixed factor. At fixed medium strength of 0.5x, the longest shoots (29 mm) obtained at medium adjusted to pH 5.0 and dispensed at 6 ml and the shortest (12.33 mm) in medium adjusted to pH 6.5 and dispensed at 7 and 8 ml per culture. At fixed strength of 1.0x**,** the longest shoots (24.7 mm) obtained in medium adjusted to pH 5.0 and dispensed at 3 and 4 ml and medium adjusted to pH 5.5 and dispensed at 4, 5 and 8 ml per culture and the shortest (12.8 mm) in medium

adjusted to pH 6.5 and dispensed at 3 and 5 ml per culture. At equal volume of 6 and 7 ml per culture equal shoot length (18.5 mm) obtained in medium adjusted to pH 5.0 and pH 5.5. Adjusting the medium to pH 5.0 and 5.5 and dispensing the medium at 4 ml per culture doubled the shoot length (22.2 and 26.7 mm) compared to medium adjusted to pH 6.5 (12.3 mm). In medium strength 1.5x, the longest shoots (22.2 mm) obtained in medium adjusted to pH 5.5 and dispensed at 3 and 4 ml and in medium adjusted to pH 6.5 and dispensed at 6 ml and the shortest shoots (12.8 mm) in medium adjusted to pH 5.5 and dispensed at 7 ml. In medium adjusted to pH 5.0 equal shoot length (18.3 mm) obtained at all volumes per culture. In medium strength 2.0x, the longest shoots (22.2 mm) obtained in medium adjusted to pH 5.5 and dispensed at 3 ml per culture and the shortest shoots (8.3 mm) in medium adjusted to pH 6.5 and dispensed at 8 ml.

Of all treatments the highest total (6750 shoots) obtained using double strength (2.0x) medium adjusted to pH 5.0 and dispensed at 4 ml per culture and the lowest total (429 shoots) using half strength medium (0.5x) adjusted to pH 5.0 and dispensed at 7 ml per culture (Table, 3.29). Keeping one of these factors fixed the combination of the other two factors which resulted in highest and lowest total per liter varied depending on the level of the fixed factor. The highest total shoots per liter of MS at strength 0.5x, 1.0x and 1.5x was 3000, 4000 and 6000 shoots all obtained in medium adjusted to pH 5.0 and dispensed at 3 ml and the highest total at strength 2.0x (6750 shoots) obtained in medium adjusted to pH 5.0 but dispensed at 4 ml per culture. However, the lowest totals obtained at different pH adjustments and medium volume per culture. The lowest total shoots of medium strength 0.5x (429 shoots) obtained in medium adjusted to pH 5.0 and dispensed at 7 ml per culture while the lowest total of strength 1.0x (1500 shoots) obtained in medium adjusted to pH 5.5 and dispensed at three different volumes (4, 6 and 8 ml) and the lowest of strength 1.5x (2250 shoots) obtained in medium adjusted to pH 5.5 and dispensed at 8 ml and medium

adjusted to pH 6.5 and dispensed at two different volumes (6 and 8 ml) per culture. The lowest of medium strength 2.0x (750 shoots) obtained in medium adjusted to pH 6.5 and dispensed at 8 ml per culture. At all media strength, increasing the volume per culture decreased the total per liter while at all medium volumes per culture increasing the medium strength increased the total per liter.

Fixing the medium strength at 0.5x, at all volumes per culture, medium adjusted to pH 5.0 produced more total than medium adjusted to pH 5.5 and 6.5 except at 3 ml per culture in which equal total (3000 shoots) obtained in medium adjusted to pH 5.0 and pH 6.5 and at 6 ml per culture in which equal total (1000 shoots) obtained in all pH adjustments. At all medium volumes per culture equal total obtained in media adjusted to pH 5.5 and 6.5 except at 3 ml per culture where pH 6.5 resulted in higher total (3000 shoots) than pH 5.5 (1000 shoots). At all pH adjustments, the highest total of medium strength 0.5x obtained by dispensing the medium at 3 ml per culture and increasing the medium volume per culture decreased the total shoots per liter. However, while in medium adjusted to pH 5.0 each 1 ml increase in medium volume up to 7 ml per culture (lowest total) resulted in gradual significant decrease in total, dispensing the medium at 8 ml per culture resulted in total shoots two times higher than that of 6 ml and three times more than that of 7 ml. In medium adjusted to pH 5.5 and 6.5 the total shoots fluctuated at each 3 to 4 ml increase in volume per culture. Similar, at fixed strength of 1.0x, the highest total (4000 shoots) obtained in medium adjusted to pH 5.0 dispensed at 3 and 6 ml per culture and the lowest (2143 shoots) at 7 ml while in medium adjusted to pH 5.5 the highest total (3000 shoots) obtained in medium dispensed at 3 ml per culture and lowest total (1500 shoots) obtained in three different medium volumes per culture (4, 6, and 8 ml). In medium adjusted to pH 6.5, three medium volumes per culture (3, 6 and 8 ml) resulted in the highest total (3000 shoots) and one medium volume per (7 ml) resulted in the lowest total (1714 shoots).

Increasing the medium pH from 5.0 to 5.5 decreased totals at all medium volumes per culture except 8 ml in which pH 6.5 resulted in higher total than pH 5.0 and pH 5.5 while increasing pH from 5.5 to 6.5 increased total at three medium volumes per culture (4, 6 and 8 ml) and equal total obtained in both pH adjustments on the other three volumes per culture (3, 5 and 7 ml). Increasing the medium volume up to 5 ml per culture decreased the total shoot per liter in media adjusted to pH 5.0 and pH 6.5 and the total fluctuated afterward.

At fixed strength of 1.5x, the highest total (6000 shoots) obtained in medium adjusted to pH 5.0 and dispensed at 3 ml per culture while the lowest total (2250 shoots) obtained in medium adjusted to pH 5.5 and dispensed at 8 ml and medium adjusted to pH 6.5 and dispensed at 6 and 8 ml per culture. Fixing the strength at 1.5x and adjusting to pH 5.0, increasing medium volume per culture decreased total shoots per liter and the lowest total obtained in medium dispensed at 7 ml per culture. However, contrary to medium strength 0.5x and 1.0x, the total shoot per liter in medium adjusted to pH 6.5 increased as the medium volume per culture increased up to 5 ml then decreased afterwards. In medium adjusted to pH 5.5, the total was not affected by medium volume up to 5 ml per culture and the highest total (3857 shoots) obtained at 7 ml per culture. At all medium volumes per culture, increasing the medium pH from 5.0 to 5.5 decreased the total shoots obtained per liter except in media dispensed at 7 ml per culture in which equal total obtained at both pH adjustments. Increasing the medium pH from 5.5 to 6.5 decreased the total if the medium dispensed at 7 ml per culture and equal total on both pH adjustments obtained in medium dispensed at 3, 6 and 8 ml but on the contrary increased the total in medium dispensed at 4 and 5 ml per culture.

A fixed strength of 2.0x, the highest total (6750 shoots) obtained in medium adjusted to pH 5.0 and dispensed at 4 ml per culture while the lowest total (750 shoots) obtained in

medium adjusted to pH 6.5 and dispensed at 8 ml per culture. In media adjusted to pH 5.0 and pH 5.5 the total shoots decreased from 6000 shoots in medium dispensed at 3 ml per culture to 4000 and 2500 shoot in medium dispensed at 6 ml per culture but increased afterward while in medium adjusted to pH 6.5 the total shoots decreased at each increase in medium volume per culture from 4000 shoots at 3 ml per culture to 750 shoot at 8 ml per culture. At all medium volumes per culture, increasing the medium pH from 5.0 to 5.5 decreased the total shoots per liter except at 3 and 8 ml per culture in which equal total obtained at both pH adjustments and increasing the medium pH from 5.5 to 6.5 decreased the total except in medium dispensed at 6 ml per culture in which equal total obtained in both pH adjustments.

Using of double strength MS (2.0x) dispensed at 3 ml and half strength MS (0.5x) dispensed at 8 ml resulted in the highest (RM 273.92) and the lowest (RM 104.07) total cost per liter respectively (Table, 3.29). Generally, at all media strength increasing the medium volume per culture decreased the total cost and at all medium volumes per culture using of higher medium strength increased the total cost per liter. For instant, the highest cost at media strength 0.5, 1.0, 1.5x and 2.0x was RM 261.92, RM 265.92, RM 269.92 and RM 273.92 obtained when the media were dispensed at 3 ml while dispensing the media at 8 ml per culture resulted in the lowest cost RM 104.07, RM 108.07, RM 112.07and RM 116.07 respectively The cost per shoot, on the other hand, ranged from RM 0.03 to RM 0.27. Nine of the tested 72 combinations resulted in the lowest cost per shoot (RM 0.03). In all of these 9 combinations, the medium strength was higher than 1.0x, dispensed at volume larger than 6 ml per culture. However, while seven was adjusted to pH 5.0, two were adjusted to pH 5.5. None of media strength adjusted to pH 6.5 resulted in shoot cost lower than RM 0.04. Four of the 72 combinations resulted in shoot cost RM 0.27 and in all of them the medium strength was 0.5x but at different pH adjustments and medium

volumes per culture. However, the combinations of media strength, medium volumes per culture and pH which resulted in the lowest cost per shoot (RM 0.03) had different total cost and total shoots per liter ranged from RM 112.07 to RM 210.75 and total of 3000 to 6750 shoots per liter (Table, 3. 29).. Partitioning the cost of items during multiplication stage using MS 2.0x adjusted to pH 5.0 and dispensed at 4 ml per culture (Figure, 3.18) indicated that the major cost factor was electricity of incubation room (46 %) followed by labor (21 %) and culture tubes cost (18%).

In almost all previous *in vitro* multiplication studies of pineapple, the effect of factors such medium strength, pH adjustments and medium volume per culture were in generally ignored. The medium strength was fixed at full strength (1.0x) MS, pH adjusted 5.7 and dispensed at 20 ml per culture. In few cases, adjustment of the medium pH to 5.0 (Fitchet, 1990a) and 6.5 (Teixeira *et al*., 2006) and on others dispensing of the medium at 10 (Hamad and Taha, 2003), 20 (Teixeira *et al*., 2006), 25 (Aydeih *et al*., 2000), 30 (Be and Debergh 2006), 40 (Sripaoraya *et al*., 2001b) and 50 ml (Sripaoraya *et al*., 2003) per culture were reported but the effect of different levels of both pH and media volume was not compared neither individually nor in combination with other factors. This study demonstrated that medium volume as low as 4 ml of doubled strength (2.0x) and as low as 6 ml of full strength (1.0x) MS per culture could be used for multiplication of pineapple and adjusting the pH to 5.0 induced better shoot formation than other pH adjustments. In fact, the shoot formation per explant at 4 ml of doubled strength and 6 ml per culture of full strength MS (Table, 3 28) was higher than that reported using 10, 20 and 25 ml and comparable to multiplication rate using 30 and 50 ml of full strength (1.0x) MS medium. Using half strength MS, Omokoio *et al*. (2001) reported a rate of 6 shoots and Teng (1997) and Zepeda and Sagawa (1981) reported a rate of 3 shoots per explants. Full strength MS was better than half strength (Khan *et al*. (2004) but the rate in both strengths was low, 4

and 3 shoots respectively. On the contrary, this study showed that half strength (0.5x) MS is not an option for multiplication of Moris pineapple. At all medium volumes per culture and pH adjustments (Table, 3.28), the lowest rate obtained using half strength medium. The other medium strengths (2.0x, 1.5x and 1.0x) could possibly be used provided the medium adjusted to proper pH and dispensed at proper volume per culture. Surprisingly, adjusting of full strength (1.0x) MS medium, the most commonly used strength of MS, to pH 5.5 which very close to the most common pH adjustment (pH 5.7) resulted in the lowest shoot formation per explant at all medium volumes per culture. The rate at medium strength 1.5x and 2.0x was two times higher than that at strength 1.0x (Table, 3.28). MS at strength 1.0x was better than other media strength only if the medium adjusted to pH 6.5 and dispensed at volume larger than 5 ml per culture and if adjusted to pH 5.0 and dispensed at 8 ml. In media adjusted to pH 5.5, medium strength 2.0x was the best strength if the media dispensed at 3, 4, 5 and 8 ml per culture while medium strength 1.5x was the best strength if the media dispensed at 6 and 7 ml per culture. However, if the media adjusted to pH 6.5 and dispensed at 4, 5 and 7 ml per culture, the best rate obtained in medium strength 1.5x and if dispensed at 6 and 8 ml, the best rate obtained in medium strength 1.0x while at all medium volumes except 3 ml per culture, medium strength 0.5 resulted in the lowest shoot formation. Adjusting to pH 6.5 (Teixeira *et al*., 2006) and pH 5.0 (Fitchet, 1990a) were reported and although assumingly both adjustments were better than pH 5.7 neither clear statement nor supporting data were provided. Both researchers used 20 ml of medium per culture and the rate of shoot formations were 13 and 7 shoots respectively. Comparable rate of 8 shoots obtained in MS strength 1.0x adjusted to pH 5.0 and pH 6.5 (Table, 3.28) using three times less amount of medium (6 and 8 ml per culture).

Shoot multiplication treatments are usually assessed by comparing of the shoot formation rate per explant. Shoot formation rate is the very important parameter to

compare the effectiveness of the treatments and indicates potential for mass production, but it is not enough for proper management of micropropagation and evaluation of the cost. Six different combinations (Table, 3.28) of medium strength, pH and medium volumes per culture resulted in the highest shoot formation (9 and 10 shoots). Selection between these treatments should not be arbitrarily or randomly made. Other parameter should be introduced to select the best of these treatments. According to shoot formation per explant, the combinations of medium strength, volumes and pH was grouped into 10 statistically different groups (Table, 3.28) while according to total shoots per liter the combinations grouped into 21 different group (Table, 3.29). Most interesting, the combination which was ranked as best according to shoot rate (medium strength 2.0x, pH 5.0 and 7 ml per culture) was placed in the fifth rank according to total per liter and one of the five combinations that were ranked as the second best according to rate per shoot was the best according to total per liter while the other four combinations placed at the eight and eleventh rank according to total per liter.

The lowest and highest shoot formation rate per explant in media dispensed at 3, 5 and 6 ml per culture was 1 and 6, 2 and 7and 2 and 8 shoots respectively and the lowest and highest rate in media dispensed at 7 and 8 ml per culture was 2 and 9 shoots (Table, 3.28). At equal volume of medium, the pH and media strength combinations which resulted in the lowest and that which resulted in the highest rate of shoot formation resulted also in the lowest and highest total shoots per liter. However, if different volumes (unequal volumes) of media were compared, this association between high rate per explant and high total shoots per liter coulds be reversed by dispensing the medium at different volumes. The medium volume per culture which resulted in higher rate produced the lowest total shoots. Dispensing the medium at smaller volume resulted in lower rate per explant (Table, 3. 28) but produced the highest total per liter of medium (Table, 3. 29). According to rate per

explant, MS strength 2.0x adjusted to pH 5.0 and dispensed at 7 ml was the best treatment resulting in the highest rate of shoot per explant (10 shoots). However, the highest total per liter obtained when the medium dispensed at 4 ml per culture (6750 shoots). Total per liter is very practical and serve the purpose of mass production more than rate per explant. Different medium volumes per culture and different media strength not only affected the rate and total but also the total cost and cost per shoot. Dispensing the medium at smaller volume per culture increased the total shoots per liter but required more culture tubes, shelving space and working hours than dispensing the medium at larger volume per culture. Dispensing the medium at 4 ml (for highest total shoots) and at 7 ml (highest shoot rate) per culture resulted in production of 6750 and 4286 shoots at total cost of RM 210.75 and RM 129.59 (Table, 3.29) and equal cost per shoot (RM 0.03). In commercial laboratory where hundreds of liters are used, compromise between saving RM 80 and production of extra 2464 shoots per liter would depend on budget and facilities available and amount and delivery time of propagules. In addition to these two treatments, other combinations of media strength, pH adjustments and medium volume per culture resulted also in lowest cost per shoot (RM 0.03) but at different total cost and total shoots per liter (Table, 3.29). Compromise would be between even larger saving (RM 99) and larger extra shoot production (3750 shoots) per liter of medium. It is interesting that the pH adjustment, a simply applied and none cost factor which always ignored during optimization of multiplication, not only resulted in drastic effect in the shoot formation rate and total shoot per liter but also in a substantial difference in total cost and cost per shoots. MS strength 2.0x adjusted to pH 5.0 resulted in 6750 shoots at total cost of RM 210 per liter of medium and RM 0.03 per shoots while adjusting the same medium to pH 5.5 and pH 6.5 reduced the total shoots to 4500 and 3750 and doubled the cost per shoot, RM 0.05 and RM 0.06 respectively (Table, 3.29). The main factors that determine commerciality of a product are

the total cost and cost per unit of production. Not only both types of cost were never reported but also the effect of medium strength and medium volume per culture on the multiplication rate was not investigated. A 30 % cost reduction using bioreactor (Escalona *et al*., 1999) was claimed, but the amount of medium used per one bioreactor cycle was too huge about 30 liters while the total shoots obtained was 3000 shoots per 45 days. Even if the other items of cost were not included, just the cost of medium per single shoot is too high.

Following the same approach of fixing the medium strength, pH adjustment and medium volume per culture which unanimously adopted in all reported pineapple studies, the highest possible multiplication would not be achieved and neither the total cost nor the cost per shoot could be minimized. A different of 0.5 in medium strength, 0.5 in medium pH and 1 ml in medium volume per culture had great impact on the shoot formation per explant (Table, 3.28), total shoot per liter of medium (Table, 3.29) depending on the combination of the other two factors. Subtracting the lowest rate of shoot formation per explant and lowest total shoots per liter from the highest and the second highest rate and total shoots indicated that using an arbitrary picked combination of combination of strength, pH and medium volume per culture, the rate of shoot formation would be less than the possibly obtainable rate of 10 shoots per explant by at least 1 to maximum of 8 shoots per explant and the total per liter less than the possibly obtainable 6750 shoots per liter by at least 750 to maximum of 6321 shoots. Substracting the rate per explant and total shoots per liter at each 0.5 increase in medium strength increase the rate by 1 to 6 shoots or decrease the rate by 1 to 4 shoots and increase the total per liter by 429 to 3000 shoots or decrease the total by 500 to 1469 shoots depending on the combination of medium strength and pH adjustments. Similar, each 1 ml increase in medium volume per culture could increase or decrease the rate per explants by 1 to 3 shoots and the total per liter by 89 to

Table (3.28). Effect of strength and pH of MS medium dispensed at different volume per culture on the in vitro shoot formation and growth of Moris pineapple after 60 days incubation.

pH	Stren	Medium volumes per culure (ml)					
	(x)	3	4	5	6	7	8
Shoots per explants							
5	0.5 x	3 efg	3 efg	3 efg	2 fg	1 g	4 defg
	1 x	4 defg	5 cdef	6 bcde	8 abc	5 cdef	7 abcd
	1.5 x	6 bcde	7 abcd	6 bcde	7 abcd	9 ab	8 abc
	2 x	6 bcde	9 ab	7 abcd	8 abc	10 a	9 ab
5.5	0.5 x	1 g	1 g	2 fg	2 fg	2 fg	2 fg
	1 x	3 efg	2 fg	3 efg	3 efg	4 defg	4 defg
	1.5 x	3 efg	4 defg	5 cdef	5 cdef	9 ab	6 bcde
	2 x	6 bcde	6 bcde	6 bcde	5 cdef	6 bcde	9 ab
6.5	0.5 x	3 efg	1 g	2 fg	2 fg	2 fg	2 fg
	1 x	3 efg	3 efg	3 efg	6 bcde	4 defg	8 abc
	1.5 x	3 efg	5 cdef	7 abcd	5 cdef	6 bcde	6 bcde
	2 x	4 defg	5 cdef	5 cdef	5 cdef	4 defg	2 fg
Shoot length (mm)							
5	0.5 x	20 abcde	22.7 abcd	20.7abcd	29 a	21 abcd	18.7 abcde
	1 x	23.7 abcd	21.7 abcd	15 bcde	18.7 abcde	19.3 abcde	18.3 abcde
	1.5 x	20.3 abcde	17.7 abcde	18.3 abcde	19.3 abcde	18.3 abcde	19.3 abcde
	2 x	17.7 abcde	19.3 abcde	16 bcde	11.7 de	18.3 abcde	19.3 abcde
5.5	0.5 x	15 bcde	23 abcd	21.3 abcd	21.3 abcd	26.3 ab	17 abcde
	1 x	19.7abcde	23.7 abcd	24 abcd	17.7 abcde	17.3 abcde	24.7 abc
	1.5 x	21.7 abcd	24 abcd	18.3 abcde	17.7 abcde	12.3 cde	20.3 abcde
	2 x	22.6abcd	15.7 bcde	12 de	17 abcde	16 bcde	16 bcde
6.5	0.5 x	16.7 bcde	19 abcde	17 abcde	22.7 abcd	12.3 cde	13.7 cde
	1 x	13.3 cde	16.3 bcde	12.3 cde	16.7 bcde	16.3 bcde	16.7 bcde
	1.5 x	17.3 abcde	15.7 bcde	15.7 bcde	21 abcd	17.3 abcde	18.3 abcde
	2 x	16.3 bcde	19.3 abcde	19 abcde	20 abcde	19.3 abcde	8.3 e

Explants were cultured at density of 3 shoots per culture in MS supplemented with sucrose at 20 g/l and BAP at 2.0 mg/l and incubated at constant temperature 25 ^{0}C and 16 hour of light for 60 days.
Means followed by same letter were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range Test.

Table (3.29). Effect of strength and pH of MS medium dispensed at different volume per culture on the in total shoots and total cost per liter of medium and cost per shoot of Moris pineapple after 60 days incubation.

pH	Stren	Medium volume per culture (ml)					
	(x)	3	4	5	6	7	8
Total shoots per liter							
5	0.5 x	3000 defghij	2250 efghij	1800 ghij	1000 j	429 j	1500 ij
	1 x	4000 cdef	3750 cdefgh	3600 cdefgh	4000 cdef	2143 fghij	2625defghij
	1.5 x	6000 ab	5250 abc	3600 cdefgh	3500cdefghi	3857 cdefg	300 defghij
	2 x	6000 ab	6750 a	4200 bcdef	4000 cdef	4286 bcde	3500cdefghi
5.5	0.5 x	1000 j	750 j	1200 j	1000 j	857 j	750 j
	1 x	3000 defghij	1500 ij	1800 ghij	1500 ij	1714 hij	1500 ij
	1.5 x	3000 defghij	3000 defghij	3000 defghij	2500 efghij	3857 cdefg	2250 efghij
	2 x	6000 ab	4500 bcd	3600 cdefgh	2500 efghij	2571defghij	3375 cdefghi
6.5	0.5 x	3000 defghij	750 j	1200 j	1000 j	857 j	750 j
	1 x	3000 defghij	2250 efghij	1800 ghij	3000 defghij	1714 hij	3000 defghij
	1.5 x	3000 defghij	3750 cdefgh	4200 bcdef	2500 efghij	2571 defghij	2250 efghij
	2 x	4000 cdef	3750 cdefgh	3000 defghij	2500 efghij	1714 hij	750 j
Total cost per liter							
	0.5 x	261.92	198.75	160.85	135.59	117.59	104.07
	1 x	265.92	202.75	164.85	139.59	121.59	108.07
	1.x5	269.92	206.75	168.85	143.59	125.59	112.07
	2 x	273.92	210.75	172.85	147.59	129.59	116.07
Cost per shoot							
5	0.5 x	0.09	0.09	0.09	0.14	0.27	0.07
	1 x	0.07	0.05	0.04	0.03	0.05	0.04
	1.5 x	0.04	0.04	0.04	0.04	0.03	0.03
	2 x	0.04	0.03	0.04	0.03	0.03	0.03
5.5	0.5 x	0.27	0.27	0.14	0.14	0.14	0.14
	1 x	0.09	0.14	0.09	0.09	0.07	0.07
	1.5 x	0.09	0.07	0.06	0.06	0.03	0.05
	2 x	0.04	0.05	0.05	0.06	0.05	0.03
6.5	0.5 x	0.09	0.28	0.14	0.14	0.15	0.15
	1 x	0.09	0.09	0.10	0.05	0.08	0.04
	1.5 x	0.09	0.06	0.04	0.06	0.05	0.05
	2 x	0.07	0.06	0.06	0.06	0.08	0.15

Explants were cultured at density of 3 shoots per culture in liquid full strength MS supplemented with sucrose at 20 g/l and BAP at 2.0 mg/l and incubated at constant temperature 25 ^{0} C and 16 hour of light for 60 days.

Means followed by same letter were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range Test.

Cost calculation was limited to variable cost items (Culture tubes, MS, sucrose, hormone, electricity for operating laminar, autoclave and incubation room, labor wages).

RM= Ringgit Malaysia (1 USA $ = RM 3.25).

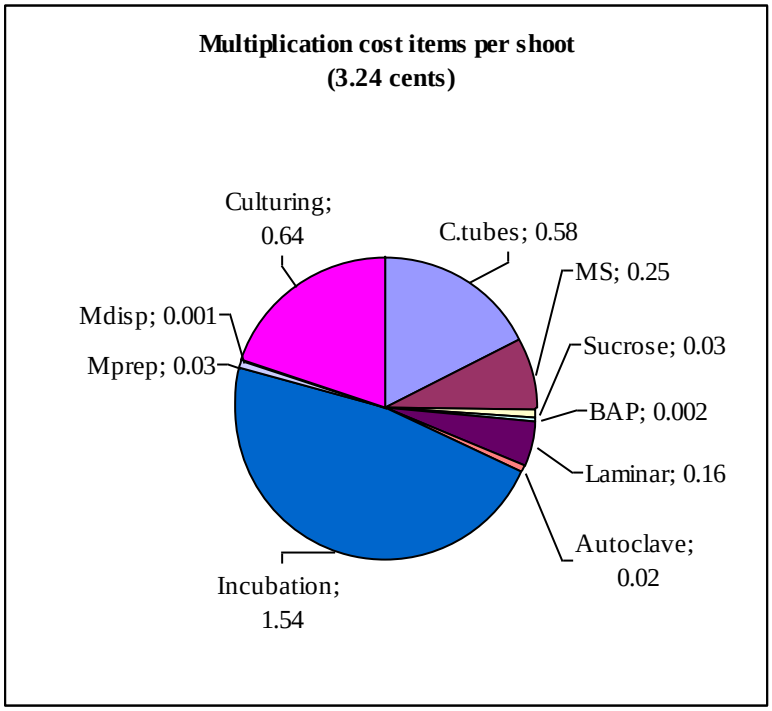


Figure (3.18). Mutltiplication cost items per shoot of Moris pineapple cultured at density of three explants per culture tube containing 6 ml of liquid full strength MS medium enriched with sucrose at 20 g/l, BAP at 2.0 mg/l and adjusted to pH 5.0. The cultures were incubated for 60 days under constant temperature 25 ^{0} C and 16 hours of light provided by cool white florescent lamps.
Mprep (Medium preparation); Mdisp (Medium dispensing); Ctubes (Culture tubes).
Culturing (separation and culturing of shoots under laminar)
Laminar, Autoclave, Incubation (electricity cost of operating laminar, autoclave and incubation room)

1357 or decreased by 200 to 2550 shoots. A 0.5 increase in medium pH could increase the rate by 1 to 4 shoots or decrease the rate by 1 to 7 shoots and the total per liter increase by 352 to 1933 shoots or decrease by 429 to 3000 shoots depending on the combination of the other factors.

3.2.14. Efffect of explants density, medium volumes and subcultures on the *in vitro* multiplication and growth of Moriss pineapple

The *in vitro* shoots formation capacity of individually separated shoots of Moris pineapple were significantly affected by medium volumes and explants density per culture and varied over subcultures (Table, 3.30). The highest shoots formation per explant (9 shoots) obtained during the third subculture using one explant in 6 and 8 ml of medium per culture and the lowest shoot formation (4 shoots) obtained at the second subculture using two explants in 4 ml and one, two and four explants in 6 ml of medium per culture. Comparing the results at fixed subculture indicated that at the first subculture, the shoots formation capacity was not affected by explants density but larger volume of medium per culture improved the shoot formation per explant. Equal shoots formation obtained at different explants density but at each explants density increasing the medium volume per culture from 4 to 6 ml increased the shoots formation per explant from 5 to 7 shoots while equal shoots formation obtained in cultures containing 6 and 8 ml of medium. At the second subculture, at all medium volumes per culture (4, 6 and 8 ml) using of three explants resulted in the highest shoot formation per explant (6, 5 and 6 shoots). However, while density of 1 and 2 explants per culture resulted in equal shoots in cultures containing 6 ml (4 shoots) and 8 ml of medium (5 shoots), in cultures containing 4 ml of medium increasing the density to two decreased the shoots formation per explants from 5 to 4 shoots. The shoots formation in cultures containing 4 and 6 ml of medium also declined

from 6 to 5 and from 5 to 4 shoots as the explants density increased from three to four explants, but remained stable (6 shoots) in cultures containing 8 ml of medium. At the third subculture, at any medium volume per culture (4, 6 and 8 ml) the highest shoots formation obtained using one explant per culture (8, 9 and 9 shoots). However, while in cultures containing 4 and 6 ml, the shoots formation capacity declined from 8 and 9 shoots at density of one explant per culture to 5 and 7 shoots per explant at density of 4 explants per culture, the explants in cultures containing 8 ml of medium maintained equal shoots formation capacity at 6 shoots per explants at density of two, three and four explants per culture. At subculture four, in cultures containing 4 ml of medium, the highest shoot formation (6 shoots) obtained using 1 and 2 explants per culture and increasing the explants density to 3 and 4 explants decreased the shoot formation to 5 shoots per explant. However, in cultures containing 6 and 8 ml, the highest shoot formation obtained using two explants (7 shoots) and the shoot formation capacity declined gradually to 6 and 5 shoots as the explants density increased to 3 and 4 explants per culture.

Dispensing the medium at 4 ml per culture resulted in the lowest shoot formation per explant at all explants density at the first subculture. However, dispensing the medium at 6 ml per culture resulted in the lowest shoot formation at the second subcultures (Table, 3.30). At the third and fourth subculture different explants density had different optimal medium volume per culture. At the third subculture, highest shoot formation at density of 2 and 3 explants (7 shoots) obtained in cultures containing 6 ml of medium and equal shoot formation (6 shoots) obtained in cultures containing 4 and 8 ml of medium while at the fourth subculture the highest shoot formation (7 and 6 shoots) obtained in cultures containing 6 and 8 ml and the lowest sboot formation (6 and 5 shoots) in cultures containing 4 ml of medium. On the other hand, the highest shoot formation at density of 1 explant (9 shoots) and 4 explants (6 shoots) at the third subcultures obtained in cultures

containing 6 and 8 ml of medium respectively while at the fourth subculture equal rate obtained (6 shoots) at all volumes. In other word, for maximum shoot formation rate, the explants density and medium volume should adjusted at different subculture.

At density of 1 explant, at all medium volumes per culture (4, 6 and 8 ml) the shoot formation per explant increased over the first three subcultures from 5 to 8 and 9 shoots and declined at the fourth to 6 shoots. At density of 2 explants, the shoot formation in cultures containing 4 ml of medium increased over the first three subcultures from 5 to 6 and remained stable at the fourth subculture. In cultures containing 6 and 8 ml of medium, the second subculture resulted in the lowest shoot formation. However, the explants regained back its shoot formation capacity abruptly in culture containing 6 ml and gradually in culture containing 8 ml during the third and fourth subculture. At density of 3 explants per culture, the shoot formation in cultures containing 4 ml of medium increased over the first two subcultures while in cultures containing 8 ml the rate decreased by the second subculture but remained stable at 6 shoots at the third and fourth subcultures. At density of 4 explants, subcultures had no effect in shoot formation of the explants incubated in 4 ml of medium per culture. But the shoot formation in cultures containing 6 and 8 ml of medium decreased over subcultures from 7 shoots at first to 5 shoots at the fourth subculture.

Average overall explants density indicated that the shoots length in culture containing 4 ml increased over the first three subcultures (15.6 to 18.0 mm) while at 6 ml the longest shoots obtained at first and third subculture (16.6 mm) and shortest at fourth (14.25 mm) and in 8 ml the shortest shoots obtained during the second subculture (15.2 mm) while equal shoot length (16.11 mm) at the other subcultures (Table, 3.30). On the other hand, average over medium volumes per culture indicated that the shoots length at density one, three and four explants increased over the first three subcultures. The longest shoot (17.0,

16.9 and 17.9 mm) obtained during the third, but the shortest shoot at density of one (13.4 mm) and 4 explants (15.4 mm) obtained during the second subculture and the shortest at density of three explants (13.4 mm) obtained at the first subculture. On contrary, the shoot length at density of two explants decreased over subcultures. The longest shoots (20.4 mm) obtained at the first and the shortest (13.2 mm) during the second subculture. Different combination of explants density and medium volumes per culture resulted in significant different in shoot length. During the first subculture, at all medium volumes (4, 6 and 8 ml) using of two explants per culture resulted in longest shoots (20.2 mm) and using of three explants in shortest shoots (13.5 mm) and at all explants density (1, 2, 3 and 4) the longest shoots obtained in 6 ml of medium (15.2, 21.3, 14.2 and 16.0 mm) while equal shoot length obtained in 4 and 8 ml of medium (14.2, 20.0, 13.1 and 15.2 mm). During the second subculture, at all medium volumes (4, 6 and 8 ml) using of four explants per culture resulted in the longest shoots (14.2, 16.0 and 16.8 mm) but the explants density which resulted in the shortest varied at different medium volumes. In cultures containing 6 and 8 ml of medium, density of one explant resulted in the shortest (14.2 mm) while in culture containing 4 ml of medium using of two explants resulted in the shortest shoots (10.7 mm). During the third subculture, at all medium volumes using of two explants per culture resulted in the shortest (16.8, 15.2 and 15.2 mm). However, while using of four explants resulted in the longest shoot (19.0 mm) in cultures containing 4 ml, equal shoot length (16.8 mm) obtained at all explants density in cultures containing 6 and 8 ml of medium. During the fourth subculture, at all medium volumes using of one explant resulted in shortest (16.8, 13.1 and 15.2 mm) and using both of two and four explants resulted in the longest shoots (18.0, 15.2 and 16.8 mm).

The highest total over four consecutive subcultures (22680 shoots) obtained using one explant and 8 ml of medium per culture and the lowest (7500 shoots) obtained using four

explants in 4 ml of medium (Table, 3.31). Equal total (about 17606 shoots) obtained at five combinations of explants density and medium volumes (1, 2 and 3 explants in 6 ml of medium and 2 and 3 explants in 8 ml of medium), equal total of 14760 shoots obtained using one explant in 4 ml and four explants in 8 ml and equal total of 9630 shoots obtained using two explants in 4 ml and four explants in 6 ml of medium per culture. However, if the experiment was run for only one, two, three subcultures, the optimal combinations of explants density and medium volumes varied at each time. At the first subculture equal total obtained at all medium volumes and explants density combinations. After two subcultures the highest total (504 shoots) obtained using three and four explants in 8 ml of medium while the lowest (240 shoots) obtained using two explants in 4 ml of medium. Statistically equal total shoots (322 shoots) obtained using one and four explants in 4 ml, one, two and four explants in 6 ml of medium and equal total shoots (405 shoots) obtained using three explants in 4 and 6 ml and one and two explants in 8 ml of medium per culture. After 3 subcultures the highest total (3780 shoots) obtained using one explant in 8 ml of medium and the lowest (1440 shoots) using two explants in 4 ml of medium and equal total of shoots (2358 shoots) obtained using one and three explants in 4 ml, two explants in 6 and 8 ml and equal total of 3003 shoots obtained using one and three explants in 6 ml and three and four explants in 8 ml of medium per culture. It is interesting that at each subculture the highest total shoots was always obtained when the medium dispensed at 8 ml per culture and the lowest total at 4 ml per culture but at different explants density.

According to both shoot formation per explant overall subcultures (Table, 3.30) and total shoots (Table, 3.31) the best combination was one explant in 8 ml of medium resulting in the highest rate (6.75 shoots) and highest total (22680 shoots) and the least combination was 4 explants in 4 of medium resulting in the lowest rate ((5 shoots) and lowest total (7500 shoots). However, the two parameters arranged the rank of the other

combinations differently. Shoot formation per explant divided the combinations into 5 groups while total shoots divided the combinations into 6 different groups. According to rate, one explant in 6 ml of medium produced more shoots than two and three explants in 6 ml and than two and three explants in 8 ml of medium. However, all of these combinations produced equal total shoots. One and three explants in 4 ml of medium resulted in equal shoot formation but according to total shoots one explant resulted in higher total than three explants. Two explants in 6 ml resulted in higher rate than in 4 ml of medium but equal total of shoots obtained in both medium volumes and three explants in 8 ml produced higher total shoots than 4 explants in 8 ml but both resulted in equal rate. Most importantly, estimation of total shoots using shoot formation rate per explant overall subcultures resulted in higher total than that estimated using shoot formation rate per explant at each subculture (Table, 3.31). The difference in two cases was over 2000 shoots. On other word, if shoots formation rate overall subcultures is used for planning and management of propagules production a problem of shortage of total propagules than expected and a surplus of money than actually allocated is most likely.To avoid shortage of money and ensure production of required total of propagules, expectation of total should be done according to shoot formation at each subculture and calculation of cost according to shoot formation overall subcultures.

Using four explants and 4 ml of medium per culture for four subcultures resulted in the lowest total cost (RM 393.17) while using one explant and 8 ml of medium resulted in the highest total cost (RM 3819.55). The lowest cost per shoot, on the other hand, was RM 0.05 obtained using four explants in 4, 6 and 8 ml of medium per culture. After three subcultures, the lowest cost of RM 0.04 per shoots obtained using 4 explants in 8 ml of medium per culture. At different subcultures, the cost per shoot ranged from RM 0.03 to RM 0.25 (Table, 3.31). For instant, at the first subculture the cost per shoot was above RM

0.03 and below RM 0.17 and after two subcultures the cost were above RM 0.04 but below RM 0.25 while the cost after three and four subcultures reduced to be within range of RM 0.04 to 0.13 and RM 0.05 to 0.17. The major three factors of total cost (Table, 3. 32) and cost per shoot (Figure, 3.19) were electricity of incubation (45 %), labor wages (19 %) and cost of culture tubes (17 %). From each 5 cents spent for production of one shoot 2.40, 1.0 and 0.9 cents were spent during incubation, paid as wages to labor and to buy the culture tubes.

Using of fixed medium volume per culture and explants density at all subcultures is the most common practice in tissue culture. Table 3.30 and 3.31 showed that at each subculture there were different optimal combinations of explants density and medium volumes per culture. Using of one fixed treatment at each cycle simplifies the procedure but optimal multiplication and shoot production could not be obtained unless the optimal factors at each cycle were determined and applied. Since, the medium volume and explant density per culture is not only important for shoot formation but also for cost of production, it is essential for valid recommendation that the optimal level of these two factors in particular be determined experimentally instead of arbitrary dispensing of the medium and arbitrary using of explants density.

Starting with 12 explants, the highest shoot formation per explant (6.75 shoots) and highst total shoots (22680 shoots) of Moris pineapple after 4 subcultures were obtained when the explants were cultured at density of one explant in 8 ml of medium per culture while the lowest rate (5 shoots) and lowest total (7500 shoots) obtained using four explants in 4 ml of medium. The effect of explants density and medium volume per culture were not reported before, but Table 3.30 and 3.31 indicated that the shoot formation rate and total were significantly affected by explants density and medium volume per culture and the optimal combination of both factors varied at each subculture. In cultures containing 4 ml

of medium, using of one and three explants resulted in highest shoot formation and four explants resulted in lowest shoot formation average overall subcultures while intermediate rate obtained using two explants per culture (Table, 3.30). In cultures containing 6 and 8 ml, using of one explant resulted in the highest shoot formation while equal average of shoot formation obtained at density of two, three and four explants. Average at esch subculture indicated that all explants density resulted in equal shoot formation at the first subculture and the shoot formation was affected mainly by the medium volume per culture. Larger medium volume per culture (6 and 8 ml) resulted in higher rate. At the second subculture increasing the explants density to three explants increased shoot formation rate. However, at the third and fourth subculture increasing explant density, on the contrary, decreased rate of shoot formation per explant. Increasing the medium volume per culture increased the rate at the third while at fourth subculture increasing the medium volume increased the shoot formation at density of 2 and 3 explants but had no effect at density 1 and 4 explants.

Previous studies showed that at density of three explants and 10 ml of medium for 60 days (Hamad and Taha, 2003), two explants and 10 ml for 42 days (Danso *et al*., 2008), 30 ml for 60 (Be and Debergh, 2006), 20 ml for 70 days (Soneji *et al*., 2002a) and one explant and 50 ml for 42 days (Sripaoraya *et al*., 2003), 25 ml for 42 days (Aydieh *et al*., 2000), 20 ml for 75 days (Teixeira *et al*., 2006) and 30 days (Fitchet, 1990a) a rate of 6, 11, 9, 12, 10, 6, 13 and 9 shoots per explant were obtained respectively. However, the effect of explants density and medium volume per culture were neither compared at each nor over several subcultures. Using of medium as low as 4, 6 and 8 ml per culture (Table, 3.30) resulted in comparable rate taking in account that different hormone treatments and cultivars were used. Although, it is not well understood, it has been noticed that in pineapple as well as other plants, the shoot formation rate varied at different subcultures

and generally declined after the fourth subculture. The rate of shoot formation of pineapple declined after 5 (Singh and Manual, 2000) and 7 subcultures (Kofi and Adachi, 1993) while Zepeda and Sagawa (1981) obtained equal rate over 4 subcultures. The decline in shoot formation over subcultures depended on the hormone treatments and cultivars (Firoozabady and Gutterson, 2003; Kofi and Adachi, 1993). To overcome decline in rate, lowering the hormone concentration over subcultures and using ‘’nursing medium’’ (hormone free) were suggested for gerbera (Vardja and Vardja, 2001), manual removal of shoot tips for Paeony (Harris and Mantell, 1992), replacing agar with gelrite for French tarragon (Mackay and Kitto, 1988) and short exposure to multiplication hormone for kiwi (Monaclean *et al*., 2000). This study indicated that using of different explants density and medium volume per culture may overcome the decline effect of subculture in pineapple. The rate of shoot formation at density of one, two and three explants in cultures containing 4 ml of medium increased over the first three subcultures and declined at the fourth while in cultures containing four explants equal shoot formation obtained at all subcultures. On the contrary, in cultures containing 8 ml of medium, the shoot formation at density of one explant per culture increased while the shoot formation at density of two, three and four explants per culture decreased over the first three subcultures.

Starting with 1 explant, Almeida *et al*. (2002) obtained a total of 2013 shoots after 5 subcultures of 30 days long each and expected that 167585 shoots could be obtained from 80 shoots after 8 subcultures. Starting with 22 shoots cultured at density of one in 20 ml of medium, Firoozabady and Gutterson (2003) reported production of 15757 shoots after 7 subcultures each 30 days. Starting with 42 shoots cultured at density of 5 shoots in 25 ml of medium 24768 shoots was produced after 4 subcultures each for 45 days (Perez *et al*., 2009). Table 3.32 showed that starting with 12 explants a comparable total of shoots (22680 shoots) could be obtained using three times less amount of medium (8 ml per

culture). Using of different medium volumes and explants density at different subcultures have not been reported before but it very likely would result in higher shoot formation and total shoot than fixed medium volume and explants density per culture at all subcultures. For Moris pineapple using of different explants density at different subcultures, 4 explants in 8 ml of medium during the first and second subculture, one explant during the third and two explants during the fourth subculture in 8 ml of medium is expected to result in shoot formation rate of 7, 6, 9 and 7 shoots and substantial increase in the total shoots (31752 shoots) and suggested for future investigation.

Following the commonly adopted practice of fixed medium volume and explants density at all subculture, 1 explant and 8 ml per culture would definitely be recommended for multiplication of Moris pineapple. It resulted in the highest shoot formation rate (6.75 shoots) and highest total shoots (22680 shoots). However, this optimal treatment is not suitable for commercial production of propagules. The total cost was too high (RM 3819.55) and cost per shoot was very expensive (RM 0.17). On the contrary, the total cost (RM 393.18) of the treatment (4 explants, 4 ml) which resulted in lowest rate (5 shoots), the total shoots (7500 shoots) was 10 times less than the highest total shoots but the cost per shoots (RM 0.05) was three times cheaper than the treatment (1 explant, 8 ml) which resulted in the highest total (22680 shoots). At any medium volumes per culture and subculture, the lowest cost per shoot using one explant per culture was always above 12 cents (Table, 3.31). At any medium volume per culture, using of two explants did not reduce the cost below 7 cents. For the cost to reach manageable level (5 cents) the explants density per culture have to be not less than three explants. In most reported studies the explants density was always less than three explants and medium volume higher than 15 ml. Little wonder that the cost was always considered as the main obstacle of pineapple micropropagation.

Compred to density of one explant and 8 ml of medium, using other combination of explants density and medium volumes per culture, although resulted in in less total shoots (15120 shoots) and lower rate of shoot formation (6.00 shoots) it required six time less budget (RM 806.51) and produced shoots at three times cheaper cost per shoot (RM 0.05). Using of 4 explants in 6 ml of medium reduced the cost per shoot three times (RM 0.05) and total cost seven times (RM 529.49) but the total shoots reduced by almost half (10080 shoots). Other combinations (3 explants in 6 and 8 ml) maintained the cost at 0.06, reduced the total cost four times (RM 995.58 and RM 1074.93) while the reduction in total shoots was 21 % (17640 and 18144 shoots). The lowest cost per shoot after 4 subcultures (RM 0.05) obtained using 4 explants in 4, 6 and 8 ml of medium per culture. But, the total shoots at these different medium volumes were 7500, 10080, and 15120 shoots and the total cost was RM 393.18; RM 529.49 and RM 806.51 respectively (Table, 3.31). A compromise between saving of RM 277.02 and extra producing of 5040 shoots and saving RM 413.35 and extra production of 7620 shoots has to be made based on budget avialable and amount of propagules to be delivered. If the target production in 8 months period is less than 7000 shoots using of 4 explants in 4 ml would achieve the target at the lowest total cost (RM 393.18). On the other hand, equal shoot rate (Table, 3.30) and total shoots (Table, 3.31) obtained using one explant in 4 ml and 4 explants in 8 ml of medium, but in term of total cost (Table, 3.31) using of one explant in 4 ml instead of 8 ml of medium is just like throwing RM 2066.22 in the garbage.

It is quite surprising that cost was considered by many as the main obstacle of commercial micropropagation of pineapple, but in the same time in all pineapple studies neither the total cost, nor the cost per shoot were reported. Few reported an expected total shoot production after several months (Firoozabady and Gutterson, 2003; Almeida *et al*., 2002; Soneji *et al*., 2002a) or year (Sripaoraya, *et al*., 2003; Zepeda and Sagawa, 1981) but

the total and rate of shoot formation at each subculture was rarely reported (Kofi and Adachi, 1993). This study demonstrated that recommendations based on rate of shoot formation and total shoots could not be adopted for commercial production. It is common sense that for any investor before adoption of any system for commercial production a feasibility study is conducted. Total cost over several and at each subculture is the key factor that determines whether the enterprise is within the investor budget and for proper management of the project. Without knowing, the rate and total shoot production and total cost and cost per shoots at each cycle of multiplication neither the feasibility nor the proper plan and management of the project could be layout. In fact, adopting of any system for commercial production require more than these obvious prerequisites. The total cost and the cost per shoot should be divided into the various cost items and the contribution of each of these cost items determined. Minimizing of the cost could be accomplished by focusing on the items that caused the highest percentage of the cost. About 45, 19 and 17 % of the multiplication cost was due to electricity of incubation, labor wages and culture tubes (Table, 3.32; Figure, 3.19). In tropical countries and during the summer in temperate zone area 45% of the total cost could be eliminated by outdoor incubation under polyethylene or saran net house. The culture tubes could be reused by decanting the old medium and dispensing new one under laminar. However, saving the cost of culture tubes would increased the laminar operation time (electricity cost for laminar operating) and working hours. The price of culture tubes, electricity cost and labor wages varied in different countries and within countries. In global world, project which is not feasible in certain countries could be very profitable on others.

Table (3.30). Effect of medium volumes and explants density per culture on the *in vitro* shoot formation and shoot length per explant of Moris pineapple over four subcultures.

MS (ml) / culture	Explants / culture	Subcultures 1	2	3	4	Average
Shoots per explants						
4	1	5 cd	5 cd	8 ab	6 bcd	6 ABC
	2	5 cd	4 d	6 bcd	6 bcd	5.25 BC
	3	5 cd	6 bcd	6 bcd	5 cd	5.5 ABC
	4	5 cd	5 cd	5 cd	5 cd	5 C
	Average	5 DE	5 DE	6.25 ABC	5.5 CD	
6	1	7 abc	4 d	9 a	6 bcd	6.5 AB
	2	7 abc	4 d	7 abc	7 abc	6.25 ABC
	3	7 abc	5 cd	7 abc	6 bcd	6.25 ABC
	4	7 abc	4 d	6 bcd	5 cd	5.5 ABC
	Average	7 AB	4.25 E	7.25 A	6 BCD	
8	1	7 abc	5 cd	9 a	6 bcd	6.75 A
	2	7 abc	5 cd	6 bcd	7 abc	6.25 ABC
	3	7 abc	6 bcd	6 bcd	6 bcd	6.25 ABC
	4	7 abc	6 bcd	6 bcd	5 cd	6 ABC
	Average	7 AB	5.5 CD	6.75 AB	6 BCD	
Shoot length (mm)						
4	1	14.33 cdef	12 ef	18 abcd	16.67 abcde	15.25 NS
	2	20 ab	10.67 f	17 abcde	18.33 abcd	16.36 NS
	3	13 def	13 def	18 abcd	17.33 abcde	15.24 NS
	4	15 bcdef	14 cdef	19 abc	18 abcd	16.61 NS
	Average	15.58 ABC	12.42 D	18 A	17.58 AB	
6	1	15.67 bcdef	14 cdef	16.67 abcde	13.33 def	14.82 NS
	2	21.33 a	14.33 cdef	15.67 bcdef	15 bcdef	16.49 NS
	3	14.33 cdef	15 bcdef	16.33 abcde	14 cdef	15.10 NS
	4	16 abcdef	16 abcdef	17.33 abcde	14.67 bcdef	16 NS
	Average	16.83 ABC	14.53 C	16.5 ABC	14.25 CD	
8	1	14.33 cdef	14.33 cdef	16.33 abcde	15.67 bcdef	15.10 NS
	2	20 ab	14.67 bcdef	15.67 bcdef	17 abcde	16.8 NS
	3	13 def	15.33 bcdef	16.33 abcde	16 abcdef	15.26 NS
	4	15 bcdef	16.33 abcde	17.33 abcde	16.67 abcde	16.39 NS
	Average	15.58 ABC	15.17 BC	16.42 ABC	16.33 ABC	

Each treatment consisted of 12 explants.
Means followed by the same letter were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range test.
Medium was full strength, liquid stationary MS medium supplemented with sucrose at 20 g/l and BAP at 2.0 mg/l. medium adjusted to pH 5.0, autoclaved for 20 minutes at 121 ^{0} C and 1.5 kg/cm^2 and dispensed into culture tubes under laminar.
Cultures incubated under constant temperature (25 ^{0} C) and 16 hours of light provided by cool white florescent lamps for 60 days and subcultured every 60 days onto fresh medium for four times.

Table (3.31). Effect of medium volumes and explants density per culture on the total shoots, total cost and cost per shoot of Moris pineapple over four subcultures

MS (ml) / culture	Explants / culture	Subcultures 1	2	3	4	B (Rate) ^{4}
Total shoots per liter						
4	1	60 m	300 kl	2400 fg	14400 b	16506.00
	2	60 m	240 l	1440 i	8640 cd	9116.00
	3	60 m	360 jk	2160 fg	10800 c	10981.00
	4	60 m	300 kl	1500 hi	7500 d	7500.00
6	1	84 m	336 kl	3024 ef	18144 ab	19882.00
	2	84 m	336 kl	2352 fg	16464 ab	18310.00
	3	84 m	420 jk	2940 ef	17640 ab	18310.00
	4	84 m	336 kl	2016 gh	10080 cd	10981.00
8	1	84 m	420 m	3780 e	22680 a	24911.00
	2	84 m	420 jk	2520 fg	17640 ab	18310.00
	3	84 m	504 j	3024 ef	18144 ab	18310.00
	4	84 m	504 j	3024 ef	15120 b	15552.00
Total cost (RM)						
4	1	10.36	50.56	251.55	2010.21	
	2	5.34	25.44	100.81	603.28	
	3	3.66	17.06	100.81	603.28	
	4	2.82	12.87	63.12	314.36	
6	1	10.67	72.82	290.32	2610.42	
	2	5.49	36.56	145.32	1015.35	
	3	3.77	24.48	121.15	846.18	
	4	2.90	18.44	72.82	435.33	
8	1	10.98	74.97	373.61	3359.99	
	2	5.65	37.64	186.96	1120.21	
	3	3.87	25.20	149.63	896.23	
	4	2.98	18.98	112.30	672.25	
Cost per shoot (RM)						
4	1	0.17	0.20	0.13	0.16	
	2	0.09	0.13	0.09	0.09	
	3	0.06	0.06	0.06	0.07	
	4	0.05	0.05	0.05	0.05	
6	1	0.13	0.25	0.12	0.16	
	2	0.07	0.13	0.08	0.07	
	3	0.04	0.07	0.05	0.06	
	4	0.03	0.06	0.05	0.05	
8	1	0.13	0.20	0.12	0.17	
	2	0.07	0.10	0.09	0.08	
	3	0.05	0.06	0.06	0.06	
	4	0.04	0.04	0.04	0.05	

Medium was full strength, liquid stationary MS medium supplemented with sucrose at 20 g/l and BAP at 2.0 mg/l. Medium adjusted to pH 5.0, autoclaved for 20 minutes at 1.5 kg/cm2 and dispensed into culture tubes under lminar. Cultures incubated under constant temperature (25 ^{0} C) and 16 hours of light provided by cool white florescent lamps for 60 days and subcultured every 60 days into fresh medium for four times.
Total shoots estimated by multiplying of average of each subculture. (Sub1 x Sub2 x Sub3 x Sub4)
B. total estimated by average overall subcultures to the power number of sub (rate/ explant) ^{4}
RM. Malaysian Ringgit (1 USA $= RM 3.25)

Table (3.32). Estimation of amount and percentage of cost of various cost items during multiplication of Moris pineapple over four subcultures.

Cost items		Subcultures				Cost	
		1	2	3	4	Total	%
		Cost per culture (RM)					
C.tubes		0.45	3.15	18.90	113.40	135.90	16.85
Medium	MS	0.19	1.34	8.06	48.38	57.98	7.19
	Sucrose	0.04	0.30	1.77	10.64	12.76	1.58
	BAP	0.00	0.03	0.16	0.97	1.16	0.14
Electricity	Autoclave	0.03	0.21	1.26	7.56	9.06	1.12
	Laminar	0.44	1.19	5.56	31.81	39.00	4.84
	Incubation	1.20	8.40	50.40	302.40	362.40	44.93
Labor	Mprep.	0.04	0.28	1.68	10.08	12.08	1.50
	Mdisp	0.08	0.58	3.50	21.00	25.17	3.12
	Culture	0.50	3.50	21.00	126.00	151.00	18.72
Total cost		2.98	18.98	112.30	672.25	806.51	100.00
Total shoots		84	504	3024	15120		
Cost per shoot per subculture.		0.04	0.04	0.04	0.04		
Total accumulative cost		2.98	21.96	134.26	806.51	965.70	
Accumulative cost per shoot		0.04	0.04	0.04	0.05		

The total shoots starting with 12 shoots at the first subculture.
The medium was full strength, liquid stationary MS supplemented with sucrose at 20 g/l and BAP at 2.0 mg/l. medium adjusted to pH 5.0, autoclaved for 20 minutes at 121 ^{0} C and 1.5 kg/cm^{2} and dispensed into culture tubes under laminar. Cultures incubated under constant temperature (25 ^{0} C) and 16 hours of light provided by cool white inflorescence lamp for 60 days and subcultured every 60 days into fresh medium for four times.
RM. Malaysian Ringgit(1 USA $ = RM 3.25)
Mprep (Medium preparation); Mdisp (Medium dispensing); C.tubes (Culture tubes).
Culture (separation and culturing of shoots under laminar)
Autoclave, Laminar, Incubation (electricity cost for operating of autoclave, laminar and incubation room).

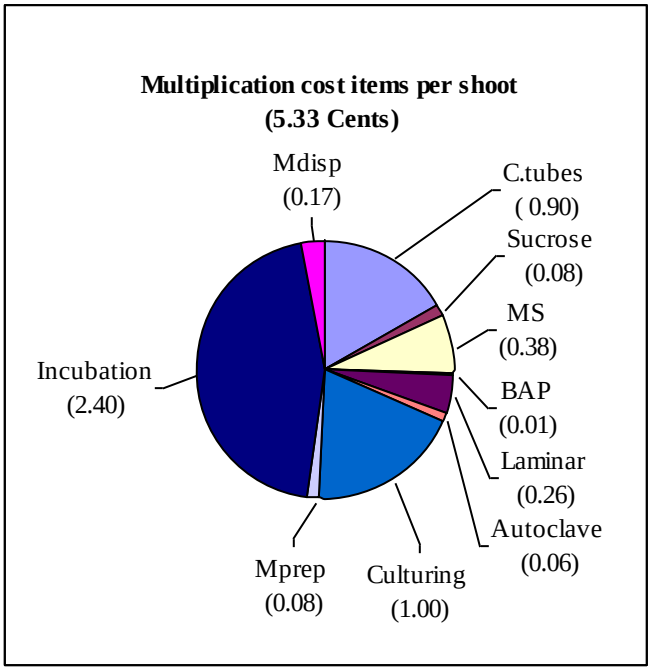


Figure (3.19) Cost per shoots (5 cents) divided into various item of the variable cost of in vitro multiplication of Moris pineapple cultured at density of 4 shoots per culture in 8 ml of liquid full strength MS medium enriched with sucrose at 20 g/l and BAP at 2.0 mg/l and incubated for 60 days under constant temperature of 25 ^{0} C and photoperiod of 16 hour of light provided by cool white inflorescence lamps.
Mprep (Medium preparation); Mdisp (Medium dispensing); C. Tubes (Culture tubes).
Culturing (separation and culturing of shoots under laminar)
Autoclave, Laminar, Incubation (electricity cost for operating autoclave, laminar and incubation room)

3.3. *In vitro* rooting of pineapples

3.3.1. Effect of auxin types, concentrations and shoot ages on the *in vitro* rooting of Smooth cayenne pineapple

Two ways analysis of variance (Table, 3.33) indicated that the rooting percentage was not affected by hormones types ($p \leq 0.63$), shoot age ($p \leq 0.06$) and their interaction ($p \leq 0.19$). The root length, on the other hand, was affected by shoot age ($p \leq 0.0003$) and interaction of the shoot age with the hormones ($p \leq 0.05$) while unaffected by the hormone types ($p \leq 0.10$). On the contrary, the root number was affected by the hormone types ($p \leq 0.0001$) and interaction of the hormone types and the shoot age ($p \leq 0.009$) while unaffected by the shoot age ($p \leq 0.32$) alone. The plantlets height, on the other hand, was under a direct independent effect of both the shoot age ($p \leq 0.0000$) and the hormone types ($p \leq 0.001$) but with no significant interaction between them ($p \leq 0.09$). Statistical analysis of each hormone as a separate experiment (Table, 3.33) indicated that rooting was affected by shoot age more than hormone and that the hormone effect was mainly related to the NAA. Shoot age significantly affected all rooting aspects except for the root length of the IBA-treated shoots and the rooting percentage of the NAA-treated shoots. NAA had no effect on two of the rooting aspects: rooting percentage ($p \leq 0.74$) and root length ($p \leq 0.35$), but induced a significant direct ($p \leq 0.000$ and 0.02 respectively) and indirect effect through interaction with age ($p \leq 0.002$ and 0.02 respectively) on the two other aspects: root number and plantlet height. IBA had no significant direct (main) or indirect (interaction) effects in three of the rooting aspects: rooting percentage ($p \leq 0.59$ and 0.85 respectively), root number ($p \leq 0.27$ and 0.58 respectively), root length ($p \leq 0.28$ and 0.22 respectively) and it only had a significant indirect ($p \leq 0.05$) effect on plantlet height through interaction with shoot age. It is interesting that although the age of the NAA-

treated shoots had neither direct ($p \leq 0.74$) nor through interaction ($p \leq 0.58$) effects on the rooting percentage, and the age of the IBA-treated shoots had neither direct or indirect significant effects on the root length, the rooting percentage of the IBA-treated shoots and the root length of the NAA-treated shoots were significantly affected by the shoot age ($P \leq 0.02$ and 0.002 respectively).

Separation of means of all combinations of shoot ages, hormone types and concentrations by Duncan test did not detect any significant differences in rooting percentage between the combinations (Table, 3.34). None of the 6-month old shoots treated with IBA produced 100 % rooting while all (100 %) of the 10-month old shoots treated with IBA at 0.5, 1.0 and 1.5 mg/l did. All of the 6-month old shoots treated with NAA at 1.0 mg/l and the 10-month old shoots treated with NAA at 1.5 $mg.L^{-1}$ produced roots. The lowest rooting percentage was 56 % obtained when the 6-month old shoots were treated with NAA at 0.5 mg/l. Although no significant differences were detected, the data could be used to estimate the confidence limit for rooting percentage. Generally, it can be said that the rooting percentage of Smooth cayenne pineapple whether hormone is used or otherwise would be around 79.3 %. In other words, in term of rooting percentage, hormone free medium could be recommended as the best treatment for Smooth cayenne. The response as measured by the other rooting parameters: root number and length and plantlet height, varied depending on the shoot age, hormone type and hormone concentrations (Table, 3.34). The roots per shoot of the 6-month old IBA treated shoots were not significantly different from hormone free (2 roots/ shoot) while that of the 10-month old were higher than hormone free but not affected by the IBA concentration. For root formation, inclusion of the IBA was more important than its concentration. The root length of the 6-month old shoots at all IBA levels except 1.5 mg/l and the plantlets height at all IBA levels except 0.5 mg/l were not significantly different from hormone free. However,

while IBA at 1.5 mg/l resulted in shorter roots (8.3 mm long) and plantlets (24.7 mm tall) than hormone free (12.3 mm long root and 34.3 mm tall plantlets), IBA at 0.5 mg/l resulted in plantlets taller (41.7 mm) than hormone free (34.3 mm) but of equal root length. That is, out of the 4 concentrations of IBA one promoted plantlets height, one inhibited root length while all had no effect on root number and rooting percentage of the 6-month old shoots. On the contrary, at all IBA concentrations, the 10-month old shoots produced more roots and taller plantlets and except at 2.0 mg/l produced longer roots than hormone free. However, while the root number and length at the different IBA concentrations were not significantly different, the differences in plantlets height were significant and increased as the IBA concentration increased, from 34.3 mm in hormone free to 76.7 mm in IBA at 1.5 mg/l and declined to 60 mm at 2.0 mg/l. All IBA concentrations promoted root formation and plantlets height and all except one promoted root length of the 10-month old shoots. On the contrary, only one of the IBA concentrations promoted plantlet height, one inhibited root length and none promoted root formation of the 6-month old shoots.

The rooting responses to NAA, on the other hand, were different from that of IBA. Compared to hormone free, NAA resulted in more roots per shoot in both ages, particularly the 6-month old shoots. 6-month old shoots treated with NAA at 1.0 mg/l produced 30 roots, the highest number of roots of all treatments. It is about three times that obtained from the 10-month old shoots and 15 times higher than that from hormone free medium. The roots per shoot decreased to 17 as the NAA concentrations increased to 1.5 and 2.0 mg/l. All NAA concentrations but 1.0 mg/l drastically decreased the root length. The root length of the shoots treated with NAA at 0.5 and 1.5 mg/l decreased from 12.3 mm in hormone free to 5.4 and 4.3 mm respectively. NAA at 1.0 mg/l resulted in the tallest plantlets (71.3 mm) in the 6-month old shoots while the height of the plantlets developed from shoots treated with NAA at 0.5 and 1.5 mg/l was not significantly different from

those of hormone free. On the contrary, the response of the 10-month old shoots to NAA treatments was in all rooting aspects better than those of hormone free and increased as the NAA increased up to 1.5 mg/l. In the presence of NAA, the 10-month old shoots produced more roots than those of hormone free but the differences in roots number per shoot between all the NAA concentrations except 1.5 mg/l were not significant. The root length increased from 9.7 mm in hormone free to 29.3 mm in response to NAA at 1.5 mg/l and the plantlets height increased from 48.3 to 129.0 mm, the tallest plantlets of all treatments. Contrary to its effect on root number per shoot where only one concentration of NAA induced a significant difference, different NAA concentrations resulted in significantly different plantlets height.

The best treatment for root formation (30 roots per explant) was the 6-month old shoots treated with NAA at 1.0 mg/l. However, the plantlets height (71.3 mm) was two times less and the root length (17.7 mm) was one and half times less than that of the 10-month shoots treated with NAA at 1.5 mg/l. The best treatment of all for tallest plantlets (129 mm) and the longest roots (29.7 mm) was the 10-month old shoots treated with NAA at 1.5 mg/l. However, the root number (10.7 roots/ explant) was three times less than that of the 6-month old shoots treated with NAA at 1.0 mg/l. The lowest root number (2 roots per shoot) was obtained from both shoot ages in hormone free medium and the 6-month old shoots treated with IBA irrespective of the concentrations. The shortest root (4.3 - 8.3 mm) was obtained from the 6-month and 10-month old shoots treated respectively with IBA at 1.5 and 2.0 mg/l and from the 6-month old shoots treated with NAA at 0.5, 1.5 and 2.0 mg/l while the shortest plantlets (24.7 mm) were obtained from the 6-month old shoots treated with IBA at 1.5 mg/l. At equal shoot age and equal concentration, except for root length of the 6-month old shoots, NAA induced better responses than IBA. In summary, the best hormone for rooting of Smooth cayenne is NAA but the concentration should be adjusted

according to shoot age at time of rooting: NAA at 1.5 mg/l used for the 10-month old shoots and at 1.0 mg/l for the 6-month old shoots. However, calculation of rooting cost (Figure, 3.20) indicated that rooting cost per shoots (not including the jars) was RM 1.01 which is definitely very expensive. The major cause of this high cost was electricity during incubation (40 %) and MS medium and labor wages (each 16 %). Using of higher explants density and lower medium volume per culture, shorter incubation, liquid medium and lower sucrose concentration would most likely reduce the rooting cost to manageable level. Jars were not included in cost because it is reusable item. However, in case of large production using of jars would increase the autoclaving time (electricity cost). Most autoclaves hold less than 100 jars at each cycle of autoclaving.

Little attention has been given to *in vitro* rooting of pineapple. All of the factors that could possibly affect rooting except the hormones were totally ignored and in almost all of the rooting studies the treatment assessments did not include all aspects of rooting responses. Previous studies suggested different rooting treatments ranging from hormone free to single and combined applications of NAA and IBA for pineapples. The use of hormone free medium for *in vitro* rooting could be justified if the assessment was limited only to rooting percentage (Table, 3.34). However, use of the other rooting responses such as root number, length and plantlets height indicated that hormone free was not a proper choice for rooting of pineapple. Shoots of different ages and different rooting parameters had different optimal hormone types and concentrations. IBA was not the proper hormone for rooting of Smooth cayenne irrespective of the shoots age and parameters. Overall for all shoot ages, IBA had no significant direct (main) or indirect (interaction) effect on rooting percentage, root number, root length and it only indirectly had a significant effect on plantlets height through interaction with shoot age (Table, 3.33). For both shoot ages, NAA was better than IBA for rooting of Smooth cayenne pineapple, but the optimal

concentration for older shoots was higher than the optimal for younger shoots. The results supported the use of NAA at 1.0 mg/l as suggested by Fitchet (1993) if the shoot age was 6 months old. However for the 10-month old shoots NAA at1.5 mg/l gave the best response for all parameters.

The main objective of the rooting stage is to improve the plantlets' survival and growth during the hardening and acclimatization stages and the selection of the best treatments should be based on which of the rooting parameters would have a great effect on the survival and growth. Rootless shoots longer than 80 mm could survive *ex vitro* rooting and acclimatization (Escalona *et al*., 1999). However, shoots longer than 80 mm could not be obtained in multiplication medium and need to pass through a month long elongation stage of two media of different BAP and GA_3 content each for two weeks (Firoozabady and Gutterson, 2003; Escalona *et al*., 1999) and incubation under high light intensity (Escalona *et al*., 2003) on medium containing antimicrobial (Gonzalez *et al*., 2005). Adoption of these elongation procedures would increase expenses and create some management problems. On the other hand, during rooting, shoots form roots and also increased in height and once they reached 35 (Dewald *et al*., 1988), 50 mm (Ko *et al*., 2006), 70 mm (Vesco *et al*., 2001) and 90 mm tall (Be and Debergh, 2006) survived acclimatization at a rate of about 95 %. Plantlets height was also found to be crucial for survival of *Euphorbia fulgens* (Zhang and Stoltz, 1989) and woody plants (Vertesy and Balla, 1987). The plantlets height seemed to be the best criteria for comparison of rooting treatments. Surprisingly, out of all researchers only Be and Debergh (2006) reported obtaining 91.0 mm long plantlets after a 60-day incubation in hormone free MS medium and 100 % survival under lath house. Table (3.34) showed that shoot elongation as well as rooting could be accomplished simultaneously by use of proper combinations of shoots age, hormone types and concentrations. Pineapple plantlets longer than 70 mm could be produced if 10-month old

shoots were treated with IBA at 0.5 and 1.5 mg/l and 6-month old shoots were treated with NAA at 1.0 mg/l. Plantlets taller than 100 mm were also obtainable if 10-month old shoots were rooted in medium enriched with NAA at 0.5, 1.0 and 1.5 mg/l. Plantlets of 129 mm tall were obtained from 10-month old shoots treated with NAA at 1.5 mg/l. *In vitro* rooting treatment which resulted in taller plantlets is a much cheaper, simpler and easier method than the two steps elongation stage.

Danso *et al*. (2008) observed that shoots that remained two months in the multiplication medium before being transferred to a rooting medium enriched with NAA at 0.5 mg/l produced two times the roots obtained from shoots remained in the multiplication medium for one month only. However, the two-month shoots lost its rooting advantages over the one-month shoots when IBA at 0.5 mg/l was used for rooting. It is not clear whether the effect was due to shoot ages or to the times the shoots remained exposed to multiplication hormones. Teng (1997) reported that during the first 5 subcultures (equal time of exposure to multiplication hormones) a specific combination of IBA and NAA was required for root induction in pineapples, while afterwards, up to the 10 th subcultures, the rooting occurred at a wide range of hormone combinations. Bhatia and Ashwath (2002) observed that rooting could even occur in the multiplication medium if the multiplication stage continued for more than 6 subcultures. These reports indicated and our results confirmed that shoot age is an important factor for *in vitro* rooting of pineapples. In other plants particularly woody species, a difference of as little as one week in shoot exposure to multiplication hormones (shoot age) could result in different rooting responses. Dogwood shoots incubated for 5, 6, 7 and 8 weeks (Sharma *et al*., 2005) and Paoeny shoots incubated for 3, 4 and 5 weeks (Harris and Mantell, 1991) in a multiplication medium before being transferred to rooting media showed that the longer the shoots remained in the multiplication medium the greater the rooting response would be. Either one or both of the

age and exposure time to multiplication medium is involved in determining the shoot rooting potential. At equal length of multiplication cycles (constant exposure time), the rooting percentage of Eucalyptus after 5 and 10 months was 16 % and 73 % respectively and after one year it reached 95 % (Trindade and Pais, 1997). Webster and Jones (1989) and Norton *et al.* (1992) reported respectively that the rooting response of M9 apple rootstock and Jonathan apple varied over 21 subcultures (shoots age). Similar, the rooting responses of *in vitro* obtained shoots of fig (Kumar *et al*., 1998) and Papaya (Rajeevan and Pandey, 1986) varied over 7 and 12 subcultures respectively.

The effect of IBA and NAA on rooting of Smooth cayenne pineapples was through different mechanisms dictated by the ages of the shoots. The shoot ages and hormone type had stronger influence in the rooting of pineapples than the hormone concentrations. It is generally well accepted that the auxins promote initiation but inhibit development of roots. However, for pineapple, the case seemed to be most likely the opposite. The root initiation (rooting percentage) was not significantly affected by the shoot age, auxin types and concentrations. The root initiation process proceeded independently of these factors. However, whether the auxin inhibited or promoted the root development (root number and length) varied at different shoot ages. In case of IBA treated shoots, except for rooting percentage, all rooting responses of the 6-month old shoots were significantly less than that of the 10-month old shoots. The different concentrations of IBA have no significant effect on root number and length of the 6-month old shoots (same as hormone free) and showed mixed effect on the plantlet height: IBA at 0.5 mg/l promoted (taller than hormone free) and at 1.5 mg/l inhibited (shorter than hormone free) and at other concentrations have no effect (same as hormone free). On the contrary, in the case of the 10-month old shoots, IBA resulted in significantly more and longer roots (promoted) than hormone free, but the different concentrations of IBA were equally effective (no significant difference). All IBA

concentrations promoted plantlets height (taller plantlets than hormone free) and the effectiveness of IBA increased as the concentration increased up to 1.5 mg/l. In the case of NAA treatments, the root length and plantlets height of the 6- month old shoots were also significantly less but the root number was significantly higher than that of the 10-month old shoots. While all NAA except at 1.0 mg/l drastically inhibited root length (less than hormone free) and had no effect (same as hormone free) on plantlets height of the 6-month old, NAA substantially promoted the root length and plantlets height of the 10-month old shoots and the NAA's effectiveness increased as the concentration increased up to 1.5 mg/l. NAA promoted the root formation of both of 6 and 10 month old shoots, but the 10-month old shoots had lower root formation capacity than the 6-month old shoots and did not respond to NAA concentrations higher than 0.5 mg/l.

In the case of the 10-month old shoots, both auxins promoted root formation (root per shoot) and were equally effective. Both at 0.5 mg/l resulted in more roots than hormone free and increasing the concentration up to 2.0 mg/l had no effect. Both also promoted root length and plantlets height but NAA was more effective than IBA. At equal concentration, NAA resulted in longer roots and taller plantlets than IBA. However, while plantlets height increased as the concentration of IBA and NAA increased to 1.5 mg/l, the root length increased as NAA increased but was not affected by IBA higher than 0.5 mg/l. In case of the 6-month old shoots, both hormones showed mixed effects on the plantlet height. IBA at 0.5 mg/l promoted and at 1.5 mg/l inhibited and at other concentrations have no effect while NAA at 1.0 mg/l promoted the plantlets height and had no effect at the other concentrations. NAA at all concentrations except at 1.0 mg/l markedly promoted the root number and drastically inhibited the root length while IBA had no effect on root number and length (same as hormone free). Since the rooting percentage (roots initial formation)

Table (3.33). Significance of main (direct) and interaction (indirect) effect of shoot ages and singly applied IBA and NAA on the *in vitro* rooting of Smooth cayenne pineapple analyzed together (A) and each hormone alone (B).

Factors	**Df**	**Rooting parameters**			
		Rooting %	Root No.	Root length	Plant height
A). Together			p values		
Shoot ages	1	0.061	0.3248	0.0003**	7.5E-10**
Hormone types	1	0.6345	0.0001**	0.1046	0.001**
Shoot ages*Hormones	1	0.1875	0.0091**	0.0493*	0.0889
Error	56				
Total	60				
B). Each hormone alone.					
IBA					
Shoot ages	1	0.0193*	0.0052**	0.0688	1.9 E-08**
IBA conc.	4	0.5919	0.2711	0.2757	0.1045
Shoot ages*IBA conc.	4	0.85	0.5845	0.2189	0.0491*
Error	20				
Total	30				
NAA					
Shoot ages	1	0.7379	0.0019**	0.0016**	1.7E-06**
NAA conc.	4	0.537	2.6E-05**	0.3499	0.0164*
Shoot age*NAA conc.	4	0.5832	0.0015**	0.1286	0.0178*
Error	20				
Total	30				

Table (3.34). Effect of shoot ages and concentrations of singly applied IBA and NAA on the *in vitro* rooting of Smooth cayenne pineapple

Conc. (mg/l)	**Shoots age and hormone types**					
	IBA			NAA		
	6 months	10months	Average	6 months	10 months	Average
Rooting %						
0	66.7 ns	75.3 ns	71 NS	66.7 ns	75.3 ns	71 NS
0.5	83.3 ns	100 ns	91.7 NS	56 ns	67 ns	61.3 NS
1	66.7 ns	100 ns	83.3 NS	100 ns	77.7 ns	88.8 NS
1.5	66.7 ns	100 ns	83.3 NS	67 ns	100 ns	83.5 NS
2	66.7 ns	83.3 ns	75 NS	89 ns	78 ns	83.5 NS
Root length (mm)						
0	12.3 bc	9.7 bc	11 NS	12.3 bc	9.7 bc	11 NS
0.5	9 bc	14.7 abc	11.8 NS	5.4 c	19 abc	12.2 NS
1	12.7 bc	19 abc	15.8 NS	17.7 abc	24.3 ab	21 NS
1.5	8.3 c	20 abc	14.2 NS	4.3 c	**29.3 a**	16.8 NS
2	9.3 bc	8.3 c	8.8 NS	8 c	29.7 a	18.8 NS
Root No						
0	2 d	2.3 d	2.2 NS	2 d	2.3 d	1.1 B
0.5	3 d	5.7 cd	4.3 NS	3 d	7.4 cd	5.2 B
1	2 d	6.3 cd	4.2 NS	**30 a**	8.3 cd	19.2 A
1.5	3 d	7.7 cd	5.3 NS	17 b	10.7 bc	13.8 A
2	2 d	4.3 cd	3.2 NS	17 b	9.3 cd	13.2 A
Plantlet hieght (mm)						
0	34.3 gh	48.3 efgh	41.3 B	34.3 gh	48.3 efgh	41.3 B
0.5	41.7 fgh	71.3 cdef	**56.5 A**	35 gh	108.3 ab	71.7 A
1	34.3 gh	69.7 def	52 AB	71.3 cdef	100.7 abc	86 A
1.5	24.7 h	76.7 cde	50.7 AB	30.3 gh	**129 a**	79.7 A
2	30 gh	60 defg	45 AB	43.3 fgh	87.3 bcd	65.3 AB

Shoots taken from stock cultures that were maintained by subculturing every 2 months on agar solidified full strength MS medium enriched with sucrose at 30 g/l and BAP at 2.23 mg/l and adjusted to pH 5.7.
The basal medium for rooting was agar solidified full strength MS medium plus sucrose at 30 g./l and adjusted to pH 5.7.
Each treatment consisted of 9 individually cultured shoots.
Data collected after 60 days of incubation under constant temperature (25^0 C) and 16 hours of light.
Means followed by same letters were not significantly different at $p \leq 0.05$ .using Duncan multiple Range Test.

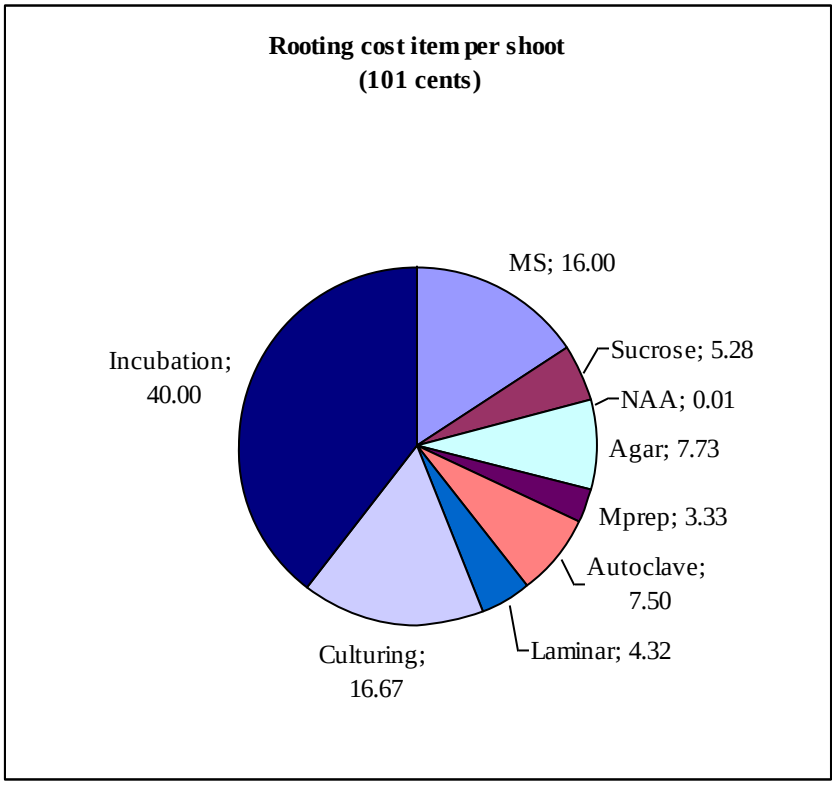


Figure (3.20) The different cost items of *in vitro* rooting of Smooth cayenne pineapple in cents.
Shoots were rooted at density of one shoot per 20 ml of agar solidified (7 g/l) full strength MS enriched with sucrose at 30 g/l and NAA at 1.0 mg/l for 60 days under constant temperature (25^0 C) and 16 hour of light provided by cool white florescent lamps.
Medium was sterilized by autoclave at 121^0 C and 1.5 kg/ cm^2 for 25 minutes.
One USA $ = RM 3.25.
Mprep (Medium preparation).
Culturing (separation and culturing of shoots under laminar)
Autoclave, Laminar, Incubation (electricity cost for operating autoclave, laminar and incubation room)

was not affected by the shoot ages, hormone types and concentration, it seemed that the 6-month old shoots had certain compounds that blocked the IBA but promoted the NAA effect on root number and those compounds decreased as the shoots increased in age.

In conclusion, investigation of the effect of different factors on the *in vitro* rooting is very important for understanding the rooting process. Agar solidified enriched with NAA at 1.0 mg/l resulted in the best rooting response, but the cost per shoot (Figure, 3.20) was unaffordable (RM 1.02). Jars were not included in cost because it is reusable item. However, in case of large production using of jars would increase the autoclaving time (electricity cost). Most autoclaves hold less than 100 jars at each cycle of autoclaving. Autoclaving the medium in large bottles (1 liter) and dispensing in culture tubes would reduce the autoclaving cost but increase the laminar operating time.

3.3.2. Effect of auxins types and concentration, MS strength and cultivars on the *in vitro* rooting of pineapple

Analysis of variance (Table, 3.35) indicated that the cultivars had significant direct effect on root number and length and plantlets height and significant indirect effect on root number and plantlets height via interaction with hormone types and with concentrations but the interaction effect on root length was not significant. The hormone types and concentrations had no direct effect except on root number per shoot. Nevertheless, both affected the plantlets height by significant interaction with cultivars and the root length by significant interaction with each other. None of the factors, cultivars, hormone and concentrations, on the other hand, had direct or indirect effect on the rooting percentage. Mean separation (Table, 3.36) show that the two cultivars reacted differently with hormone types and concentrations. The hormones effect on rooting percentage of both cultivars could be grouped into four different groups. The first group consisted of IBA at 0.5, 2.5 and NAA at 1.0 mg/l and resulted in rooting of 90.1 % of both cultivars. The second group

consisted of IBA at 1.0 and 2.0 mg/l and resulted in rooting of 90.1 % of Moris but had no effect on rooting of Smooth cayenne (equal rooting percentage as hormone free medium (61.3 %). The third group consisted of one treatment, NAA at 2.0 mg/l, and on the contrary resulted in rooting of 90.1 % of Smooth cayenne but had no effect on rooting of Moris. The fourth group included the other IBA and NAA concentrations and resulted in rooting of 61.3 % of both cultivars and was not significantly different from hormone free medium. In other words, both cultivars could be rooted in several concentrations of both hormones and IBA at 0.5 and 2.5 mg/l and NAA at 1.0 mg/l could be used for both cultivars. The rooting percentage overall concentrations indicated that IBA and NAA resulted in equal rooting percentage in case of Smooth cayenne (71.3 %) and so either one could be used for rooting of Smooth cayenne. However, in case of cultivar Moris, IBA resulted in higher rooting percentage (81.6 %) than NAA (54 %).

The root formation of the two cultivars proceeded differently. The root formation (roots/ shoot) of Moris was not affected by either hormone. At any concentration of NAA and IBA, the roots per shoot were not significantly different from that in hormone free medium. Smooth cayenne too was not affected by IBA but, using of NAA at 1.0 mg/l and above markedly increased the root formation from 2 roots obtained in hormone free to 29.7 roots/ shoot. According to root number per shoot, hormone free would be suggested for rooting of Moris while NAA at 1.0 mg/l for rooting of Smooth cayenne. The longest root of Smooth cayenne (17.7 mm) obtained in medium enriched with NAA at 1.0 mg/l while none of the IBA concentrations improved the root length of Smooth cayenne. Two of IBA concentrations (1.0 and 2.5 mg/l) had no effect on root length resulting in roots as long as hormone free medium (no effect) and the Smooth cayenne roots in the other three concentrations of IBA (0.5, 1.5 and 2.0 mg/l) were shorter roots than hormone free (inhibited) but were not significant from each other. On the contrary, the longest root of

Moris (15.3 mm) obtained in medium enriched with IBA at 0.5 mg/l. Two of IBA concentrations (1.0, 2.5 mg/l) had no effect on root length but on the other three concentrations (0.5, 1.5, 2.0 mg/l), Moris roots were significantly longer than hormone free (promoted) and were significantly different from each other. IBA inhibited the root elongation of Smooth and promoted root length of Moris. Yet, in hormone free and at equal concentration of IBA except at 0.5 mg/l, Smooth cayenne produced longer roots than Moris (Table, 3.35). NAA, on the other hand, inhibited the root elongation of both cultivars. At all NAA concentrations except at 1.0 mg/l, both of Smooth cayenne and Moris produced roots significantly shorter than hormone free (inhibited). At equal concentration except 1.0 mg/l, both of the cultivars (Smooth cayenne and Moris) produced longer roots on IBA contained than NAA contained media. At all NAA concentrations, Smooth cayenne produced root longer than Moris. Although, presence of hormone (IBA and NAA) suppressed the root elongation of both cultivars, Smooth cayenne had higher tendency of developing longer roots and the process proceeded faster than Moris. According to root length, IBA at 0.5 mg/l would be recommended for Moris and NAA at 1.0 mg/l for Smooth cayenne.

In NAA contained medium, the tallest Smooth cayenne (71.3 mm) and Moris (39.7 mm) plantlets obtained in medium enriched with NAA at 1.0 and at 0.5 mg/l respectively while in IBA contained medium the tallest Smooth cayenne (49.0 mm) and Moris (47.3 mm) obtained in medium enriched with IBA at 2.5 and at 1.5 mg/l respectively. All Smooth cayenne shoots treated with NAA resulted in plantlets taller than hormone free medium and only at one concentration (0.5 mg/l) the plantlets were shorter than IBA treated shoots but equal to hormone free medium. At three NAA concentrations (1.0, 2.0, 2.5 mg/l) Smooth caynne plantlets were taller than hormone free and than IBA treated while at one concentration (1.5 mg/l) equal plantlets height obtained at hormone free, NAA

and IBA. On the contrary all of the Moris shoots treated with NAA resulted in plantlets shorter than IBA treated shoots and only one NAA concentration (0.5 mg/l) resulted in Moris taller than hormone free medium but equal to IBA. In fact, at two concentrations of NAA (1.5 amd 2.5 mg/l) Moris were shorter than IBA and hormone free. NAA at 2.0 mg/l resulted in Moris plantlets as tall as those in hormone free medium but was shorter than those in IBA while at concentration of 1.0 mg/l equal plantlets height obtained in hormone free, NAA and IBA. For plantlets height, the two cultivars had different hormone type requirement. At equal concentrations, NAA induced taller Smooth cayenne (more effective) than IBA while IBA induced taller Moris (more effective) than NAA. Smooth cayenne was taller than Moris at one concentration of IBA (2.5 mg/l) and all NAA concentrations except 0.5 mg/l but shorter than Moris at two concentration of IBA (1.5 and 2.0 mg/l) and one concentration of NAA (0.5 mg/l) while equal height of both cultivars obtained at two concentration of IBA (0.5 and 1.0 mg/l). Irrespective of hormone treatment Smooth cayenne has higher tendency for taller plantlets. For plantlets height the two different cultivars have different hormone preferences, IBA at 1.5 mg/l for Moris and NAA at 1.0 mg/l for Smooth cayenne

Overall, both cultivars developed roots of equal length (10 mm) and plantlets of about equal height (36 mm). However, in NAA contained media Smooth cayenne had higher rooting percentage (71.4 %), more (14.5 roots) and longer (9 mm) roots and taller (46.1 mm) plantlets than Moris (54 %, 2.4 roots, 4.4 mm, 28 mm respectively) while on IBA contained media, Moris had higher rooting percentage (81.6 %) and more roots (4.4 roots) than Smooth (71.3 %, 2.8 roots respectively). The rooting percentage (71.4 %) and root length (9 mm) of Smooth cayenne in NAA and IBA contained media were equal (both hormones were equally effective). But the root number (14.5 roots) and the plantlet height (46.1 mm) in NAA contained medium was significantly higher than that (2.8 roots and

35.7 tall plantlets) in IBA contained media. NAA was more effective than IBA. On the contrary, all aspect of rooting of Moris on IBA contained media (81.6 %, 4.4 root each 7.7 mm long and 38.8 mm tall plantlets) were higher than that in NAA contained medium (54 %, 2.4 roots each 4.4 mm long and 28 mm tall plantlets). For cultivar Moris IBA was more effective than NAA. It is clear that selection of optimum hormone type and optimal concentration of that hormone varied among cultivars and depended on the parameter used for assessment. The root initiation and development in particular of the two cultivars proceeded at different rate and probably by different mechanisms. The two cultivars had different hormone type preference, NAA at 1.0 mg/l for Smooth cayenne and IBA at 0.5 and 2.0 mg/l for Moris.

Not only different cultivars had different optimal hormone treatment, but that optimal hormone treatment for the same cultivar varied at different medium strength (Table, 3.37). The medium strength affected the cultivar response to hormone types and concentrations and resulted in different rooting quality. While some combinations medium strength, hormone type and concentration induced higher rooting percentage of Smooth and other induced higher rooting response of Moris, equal rooting percentage of both cultivars obtained on same hormone treatment but different medium strength and on different hormones but same medium strength. In other word by manipulation of medium strength, one hormone treatment could be used for *in vitro* rooting of both cultivars (to develop rooting that suit both cultivars). Instead of buying two hormones, one hormone (the cheapest) could be used for both cultivars but at different strength of medium. All shoots of Smooth cayenne (100 % rooting) rooted in quarter strength while all shoots of Moris rooted on half strength MS both enriched with IBA at 2.0 mg/l. About 85.8 % of the shoots of both cultivars rooted in quarter strength-hormone free, quarter strength-enriched with IBA at 0.5 mg/l and in full strength-enriched with NAA at 1.0 mg/l. Equal rooting

percentage of 85.8 were also obtained but at different combinations. Smooth cayenne on half strength-hormone free, half strength-enriched with NAA at 1.0 mg/l and quarter strength-enriched with NAA at 1.0 mg/l and Moris on full strength-hormone free and quarter strength-enriched with IBA at 2.0 mg/l. But, this rooting percentage was not significantly different from that obtained on quarter strength hormone-free medium (the cheapest combination). Similar, about 63.1 % rooting of both cultivars obtained in full strength-enriched with IBA at 2.0 mg/l (same treatment) and the same rooting percentage of both cultivars was also obtained at different combinations. Smooth cayenne on full strength-hormone free and half strength-enriched with IBA at 2.0 mg/l and Moris on half strength-enriched with IBA at 0.5 mg/l resulted in 63.1 % rooting percentage but was significantly lower than the percentage obtained on quarter strength hormone free (cheapest combination). On the other hand, Smooth cayenne on quarter strength enriched with IBA at 2.0 and NAA at 1.0 and full strength enriched with IBA at 0.5 mg/l had higher rooting percentage than Moris while Moris in half strength enriched with IBA at 0.5 and 2.0 mg/l had higher percentage than Smooth cayenne. According to rooting percentage, IBA at 2.0 mg/l was the best for both cultivars provided that quarter strength medium used for Smooth cayenne and half strength medium used for Moris.

It is interesting that while at certain hormone treatments lowering the medium strength improved the rooting percentage of both cultivars, at other hormone treatment lowering the medium strength could decrease the rooting percentage of both, improved the rooting percentage of one cultivar but decreased the rooting percentage of the other and vice versa. Different cultivars and different rooting parameters could respond differently to changes in medium strength in accordance with types and concentrations of the hormone incorporated in the medium. On hormone free medium, lowering the medium strength from full to half strength decreased the rooting percentage of Moris while lowering the strength from full to

quarter had no effect (equal rooting percentage at both strengths). On the contrary, both reductions of medium strength increased the rooting percentage of Smooth cayenne. On IBA contained media, at both IBA concentration (0.5 and 2.0 mg/l), lowering the medium strength from full to half and from half to quarter increased the rooting percentage of Moris. On the contrary, on medium enriched with IBA at 2.0 mg/l lowering the strength from full to half had no effect and from full to quarter increased the rooting percentage while in medium enriched with IBA at 0.5 lowering the strength to half decreaed the rooting percentage and from full to quarter had no effect on rooting percentage of Smooth cayenne. On medium enriched with NAA at 1.0 mg/l, lowering the medium strength from full to half and from half to quarter had no effect on the rooting percentage of Smooth cayenne. But both reductions of medium strength decreased the rooting percentage of Moris. It is clear that reduction of medium strength was beneficial to rooting process in some cases but detrimental at others.

The highest root number per shoot (12.8 and 10.0 roots) of both cultivars obtained in MS full strength enriched with NAA at 1.0 mg/l. Compared to quarter strength hormone free, full strength increased the root number per shoot of Smooth cayenne 4 times, but increased the root number of Moris 2.5 times only. On the other hand, reducing the strength of the medium enriched with NAA at 1.0 mg/l from half to quarter reduced the root number of Smooth cayenne 2 times but that of Moris 5 times. In hormone free and media enriched with IBA at 0.5 mg/l, the root number of Smooth cayenne was not affected by medium strength. However, decreasing the medium strength of hormone free medium from full to half strength decreased the root number while in medium enriched with IBA at 0.5 mg/l increased the root number of Moris. In media enriched with IBA at 2.0 mg/l, decreasing the medium strength from full to half decrease the root number of Smooth cayenne but increased the root number of Moris cultivar. On the other hand, in both

cultivars at any hormone treatments, quarter strength medium resulted in longest and full strength on shortest roots but each cultivar had different optimal hormone for longest roots. Smooth produced the longest roots (27.7 mm) in quarter strength medium enriched with NAA at 1.0 mg/l and Moris (64.7 mm) in quarter strength enriched with IBA at 0.5 mg/l. Either cultivar could produce longer roots than the other depending on the medium strength and hormone treatments. At half strength of hormone free medium and at any strength in medium enriched with NAA at 1.0 mg/l, Smooth cayenne produced longer roots than Moris. However, at quarter and half strength in medium enriched with IBA at 0.5 and at 2.0 mg/l, Moris produced longer roots than Smooth cayenne. In full strength-hormone free and full strength enriched with IBA at 0.5 and 2.0 mg/l, Smooth cayenne and Moris had equal root length. Compared to quarter hormone free, (the cheapest treatment) addition of IBA at 0.5 mg/l had no effect on root length of Smooth (18 to 20 mm) but increased that of Moris 1.5 times (45.3 to 64.7 mm) while addition of NAA at 1.0 mg/l increased the root length of Smooth cayenne 1.5 times (18 to 27.7 mm) but decreased that of Moris 3.0 times (45.3 to 17.3 mm). Overall, the root formation process, initiation (rooting percentage) and development (root number and length) of Smooth cayenne is more responsive to hormone type than Moris while the root formation process of Moris is more sensitive to change in medium strength than Smooth cayenne.

The tallest plantlets of Smooth cayenne (99.2 mm) obtained in full strength enriched with NAA at 1.0 mg/l while the tallest of Moris (58.3 mm) obtained in quarter strength enriched with IBA at 2.0 mg/l. At half and quarter strength in media enriched with IBA at 0.5 and 2.0 mg/l and in hormone free media, Moris plantlets were taller than Smooth cayenne while in medium enriched with NAA at 1.0 mg/l Smooth cayenne plantlets were taller than Moris. For both cultivars, reducing the medium strength of the medium enriched with NAA at 1.0 mg/l from full to quarter strength decreased the plantlets height while on

medium enriched with IBA at 0.5 mg/l had no effect. However, in medium enriched with IBA at 2.0 mg/l, reducing of strength from full to quarter increased the height of both cultivars while on hormone free medium, decreased the height of Smooth but had no effect on Moris. Compared to plantlet height on quarter strength hormone free medium (31 mm tall), all treatments resulted in significantly taller Smooth cayenne (taller than31 mm) and the best treatment (full strength enriched with NAA at 1.0 mg/l) tripled the Smooth cayenne plantlet height (99 mm). All treatments resulted in Smooth cayenne taller than 40 mm, but only three treatments resulted on Smooth cayenne taller than 60 mm and only one treatment resulted in plantlets taller than 90 mm. Similar, in case of Moris, three treatments resulted in shorter, seven treatments in equally tall and only two treatments resulted in Moris plantlets taller than that obtained in quarter strength hormone free medium (50.3 mm). However, although the improvement in Moris height statistically significant practically negligible, less than 10 mm (50 to 58 mm). All treatments (combination of hormone types, concentrations and media strength) except two resulted in Moris plantlets shorter than 40 ml and none resulted in Moris plantlets taller than 60 mm (Table, 3.37). Hence, for taller Moris plantlet no hormone treatment is needed and reduction of medium strength to quarter is favored over full strength for low total cost. However, optimization of hormone and medium strength did not lower the cost of rooting per shoot. In both cultivars, the rooting cost per shoot at the optimal rooting treatment was 91 cents (Figure, 3.21). Electricity during the incubation period was the major cost factor (44 %), followed by labor wages for culturing (19 %) and culture tubes (17 %).

Although, factors such as hormone types and concentrations, medium strength and cultivars are expected to affect the rooting responses, only very limited and insufficient comparison of hormones were reported in the previous studies while the cultivars effect was completely ignored. Analysis of variance of rooting data from full strength MS

(Table, 3.35) indicated that all rooting parameters were mainly affected by cultivars more than the hormones. The cultivars affected the root formation per shoot directly and via interaction with each and with both of the hormone types and concentrations and affected the plantlets height directly and via interaction with hormone types and concentrations but the interaction of the three factors were not significant. On the other hand, although the hormone types and concentrations interacted significantly with each other and with cultivars, there were no significant different between hormone types and between concentrations in any rooting aspects except the root number per shoot. The two cultivars, Smooth cayenne and Moris showed a different and distinctive rooting response to hormone types, concentration and medium strength. In full strength medium (Table, 3.36) the highest rooting percentage, longest roots and tallest plantlets of Moris was obtained in IBA contained media while in both hormones (IBA and NAA) the root number was not significantly different from hormone free medium. On the contrary, both of the two hormones could be used for inducing highest rooting percentage of Smooth cayenne but neither was suitable for longest roots. Root length of Smooth cayenne in hormone free medium was longer than all of the IBA concentrations and all except one concentration of NAA. Contrary to Moris, the highest root number and tallest plantlets of Smooth cayenne obtained in NAA contained medium. In hormone treatments at different MS strengths (Table, 3.37), IBA at 2.0 mg/l in quarter strength MS was the best hormone treatment for highest rooting percentage of Smooth cayenne while best hormone for the other three rooting parameters was NAA at 1.0 mg/l but at different medium strength. Full strength for more roots and taller plantlets and quarter strength for longer roots. On contrary, NAA at 1.0 mg/l in full medium strength was the best for more root formation per explant of Moris cultivar while IBA was the best hormone for the other three rooting parameter but at different strength and different concentrations. IBA at 2.0 mg/l in half strength MS for

higher rooting percentage and in quarter strength for taller plantlets while IBA at 0.5 mg/l in quarter strength medium for longer roots.

Hormone free MS medium was suggested for rooting of Smooth cayenne (Ko *et al*., 2006; Be and Debergh, 2006; Wakasa, 1989), Phuket (Sripaoraya *et al*., 2003; 2001a), Perola (Almeida *et al*., 2002; 1997) and Red Spanish (Kiss *et al*., 1995). Using of NAA was suggested for *in vitro* rooting of Queen and Smooth cayenne (Aydieh *et al*., 2000; Fitchet, 1990a) and IBA for rooting of Madhupur (Akbar *et al*., 2003), Queen (Devi *et al*., 1997; Bordoloi and Sarma, 1993) and Mauritius (Fernando, 1986). On the other hand, Bhatia and Ashwath (2002) doubted the use of hormone free for rooting of Smooth cayenne and Akbar *et al*. (2003) reported that Madhupur pineapple could not root in hormone free and in NAA contained media. Using combination of IBA and NAA, Rahman *et al.* (2001) observed no differences in rooting responses of Giant Kew and Khluna pineapple. Other researchers used two (Almeida *et al*., 1997), three (Dewald *et al*., 1988) cultivars during multiplication, but in spite of difference in cultivar multiplication rate, their investigation did not extend to include the difference on the *in vitro* rooting of the the cultivars. Using of MS at half (Khan *et al*., 2004; Akbar *et al*., 2003; Almeida *et al*., 2002; Singh and Manual, 2000; Khatun *et al*., 1997; Devi *et al*., 1997; Bordoloi and Sarma, 1993; Firoozabady and Gutterson, 2003) and full strength (Be and Debergh, 2006; Bhatia and Ashwath, 2002; Rahman *et al*., 2001; Almeida *et al*., 1997) during rooting of pineapple have been reported. However, in all but one report (Soneji *et al*., 2002a) no details were provided. Soneji *et al*. (2002a) demonstrated that half strength MS resulted in equal root number and length but higher rooting percentage of Queen pineapple than full strength MS and White formulation. Our results confirmed their findings and proved that the medium strength could even be reduced further to quarter. However, the beneficial effect of medium strength reduction depended on the cultivars, hormone treatments and

rooting parameters. Optimum treatment for rooting of one cultivar could not be generalized for others and the best rooting treatment varied not only between the cultivars but also for each rooting parameter of the same cultivar (Table, 3.36 and 3.37). The suitability of hormone type, concentration and medium strength for *in vitro* rooting depended on which rooting parameters were used for assessment. None of the treatment was best for all rooting parameters.

Since the main goal of *in vitro* rooting is to ensure plantlets survival during acclimatization, selection of the best rooting treatment should be based on the rooting parameters that had strong correlation with plantlets survival. Escalona *et al*. (1999) demonstrated that for rootless ahoots the longer shoots had higher percentage of survival during *ex vitro* acclimatization. Rooted shoots of 35 (Dewald *et al*., 1988), 50 mm (Ko *et al*., 2006), 70 mm (Vesco *et al*., 2001) and 90 mm tall (Be and Debergh, 2006) survived acclimatization at a rate of about 95 %. If the minimum plantlets height for survial of rooted Moris was 40 mm, all treatments except half strength MS hormone free and quarter strength enriched with NAA at 1.0 mg/l resulted in plantlets taller than 40 mm. Hence quarter strength MS hormone free would be recommended for Moris. However if the minimum height was 60 mm, none of the treatments resulted in Moris taller than 50 mm and hence none of the treatment could be recommended and further investigations and other factors should be tested to improve the rooting of Moris. In case of Smooth cayenne, all treatments except hormone free medium resulted in plantlets taller than 40 mm and all treatments except hormone free medium could be recommended for rooting. However, only 3 treatments resulted in Smooth cayenne taller than 60 mm and one treatment in Smooth cayenne taller than 90 mm. None of the medium strength enriched with IBA resulted in Smooth cayenne taller than 60 mm while NAA at1.0 at all strength did. Quarter strength would be favored over full strength. For plantlets taller than 90 mm, NAA at 1.0

Table (3.35) Significant of main and interaction effect of cultivars, auxin types and concentrations on *in vitro* rooting of pineapple

Factors	**df**	**Rooting parameters**			
		Rooting %	Root No.	Root length	Plantlet height
Cultivars	1	0.6238	5.5E-09**	0.0032**	6.3E-07**
Hormones	1	0.0708	4.1E-08**	0.0567*	0.9638
Concentrations	5	0.2528	7.2E-06**	0.0996	0.1911
Cultivars*Hormones	1	0.0676	3.8E-12**	0.3959	0.0010**
Cultivar*Concentrations	5	0.8219	5.8E-06**	0.1659	0.0172*
Hormones*Concentrations	5	0.1915	9.5E-07**	0.0211*	0.3053
Culti*Horn*Conc	5	0.6809	6.0E-05**	0.6701	0.1751

Table (3.36) Effect of cultivars, auxin types and concentrations on the *in vitro* rooting of pineapple

Concentration (mg/l)	**Auxin types and cultivars**					
	IBA			NAA		
	Smooth	Moris	Average	Smooth	Moris	Average
Rooting %						
0	61.3 ab	66.7 ab	64 ABC	61.3 ab	66.7 ab	64 ABC
0.5	83.3 a	100 a	91.7 AB	56 ab	45 ab	50.5 BC
1	66.7 ab	77.7 a	72.2 ABC	100 a	89 a	94.5 A
1.5	66.7 ab	67 ab	66.8 ABC	67 ab	11.7 b	39.3 C
2	66.7 ab	100 a	83.3 AB	89 a	56 ab	72.5ABC
2.5	83.3 a	78 a	80.7 ABC	55.7 ab	55.3 ab	55.5 ABC
Root number						
0	2 c	3.3 c	2.7 B	2 c	3.3 c	2.7 B
0.5	2.7 c	6.3 c	4.5 B	3 c	1.4 c	2.2 B
1	2 c	3 c	2.5 B	29.7 a	4.3 c	17 A
1.5	3 c	3.7 c	3.4 B	17.3 b	0.4 c	8.9 AB
2	2.3 c	6 c	4.2 B	16.7 b	1.4 c	9 AB
2.5	5 c	4 c	4.5 B	18.3 b	3.7 c	11 AB
Root length (mm)						
0	10.3 abc	4.7 cd	7.5 ABC	10.3 abc	4.7 cd	7.5 ABC
0.5	9 abcd	15.3 ab	12.2 AB	5.4 cd	3 cd	4.2 C
1	12.7 abc	4.7 cd	8.7 ABC	17.7 a	10.3 abc	14 A
1.5	8.3 abcd	8 bcd	8.2 ABC	4.3 cd	0.4 d	2.4 C
2	9.3 abcd	9.3 abcd	9.3 ABC	8 bcd	4 cd	6 BC
2.5	12 abc	5 cd	8.5 ABC	6.3 bcd	4 cd	5.2 BC
Plantlet hieght (mm)						
0	34.3 cd	30 cd	27.2 NS	34.3 cd	30 cd	27.2 NS
0.5	41.7 bcd	40 bcd	40.9 NS	35 cd	38.7 bcd	36.9 NS
1	34.3 cd	36 cd	35.2 NS	71.3 a	28 cd	49.7 NS
1.5	24.7 cd	47.3 bc	36 NS	30.3 cd	20.3 d	25.3 NS
2	30 cd	44 bcd	37 NS	43.3 bcd	30.3 cd	36.8 NS
2.5	49 bc	35.3 cd	42.2 NS	59.7 ab	22 d	40.9 NS

Data were mean of 9 explants cultured on agar solidified full strength MS medium enriched with sucrose at 20 g/l and pH adjusted to 5.7 for 60 days.
Means of the same parameter followed by same letters were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range test.

Table (3.37). Effect of hormones types, concentration, MS strength and cultivars on the *in vitro* rooting of pineapple

Horm.	Conc.	MS	Pineapple cultivars					
			Smooth	Moris	Average	Smooth	Moris	Average
			Rooting %			**Root No.**		
Free	0	Quarter	83.3 ab	89 ab	86.2	3 e	4 de	3.5 BC
		Half	83.3 ab	30 c	56.7	3 e	2 e	2.5 C
		Full	61.3 abc	78 ab	69.6	1.7 e	4 de	2.9 BC
IBA	0.5	Quarter	83.3 ab	89 ab	86.2	2 e	5 cde	3.5 BC
		Half	50.3 bc	59 abc	54.7	2 e	4 de	3 BC
		Full	83.3 ab	44.7 bc	64	3 e	2 e	2.5 C
	2.0	Quarter	100 a	78 ab	89	2 e	3 e	2.5 C
		Half	66.7 abc	100 a	83.3	3 e	5 cde	4 BC
		Full	72.3 abc	56 abc	64.2	3.7 de	3 e	3.3 BC
NAA	1	Quarter	91.7 ab	44.7 bc	68.2	7.3 bc	2 e	4.7 BC
		Half	91.7 ab	44.7 bc	68.2	6.7 cd	4 de	5.4 B
		Full	90.1 ab	89 ab	89.5	12.8 a	10 ab	11.4 A
		Root	**Root length (mm)**			**Plantlet height**		
	0	Quarter	18 de	45.3 ab	31.7 A	31.7 e	50.3 bcde	41 BC
		Half	20.7 de	15.3 e	18 ABC	39.7 cde	29.3 e	34.5 C
		Full	11.1 e	13.7 e	12.4 BC	39.6 cde	46.3 bcde	43 BC
IBA	0.5	Quarter	20 de	64.7 a	34.8 A	40.3 cde	51 bcde	45.7 BC
		Half	14 e	24 de	19 ABC	36.7 de	47.3 bcde	42.2 BC
		Full	9 e	10.7 e	9.8 C	41.7 cde	47.3 bcde	44.5 BC
	2.0	Quarter	11.7 e	43 abc	27.3 AB	38.3 de	58.3 bcd	48.3 BC
		Half	19 de	36.3 abcd	27.7 AB	38.3 de	56.7 bcde	47.5 BC
		Full	8.3 e	10.3 e	9.3 C	43.8 cde	45 cde	44.4 BC
NAA	1	Quarter	27.7 bcde	17.3 de	22.5 ABC	71.3 b	35.7 de	53.5 BC
		Half	23 de	14.7 e	18.8 ABC	65.7 bc	47.7 bcde	56.7 AB
		Full	25.6 cde	15.3 e	20.5 ABC	99.2 a	49 bcde	74.1 A

Data represent mean of 9 explants cultured on agar solidified MS medium enriched with sucrose at 20 g/l and pH of 5.7 after 60 days of incubation.
Means of the same parameter followed by same letter were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range test.

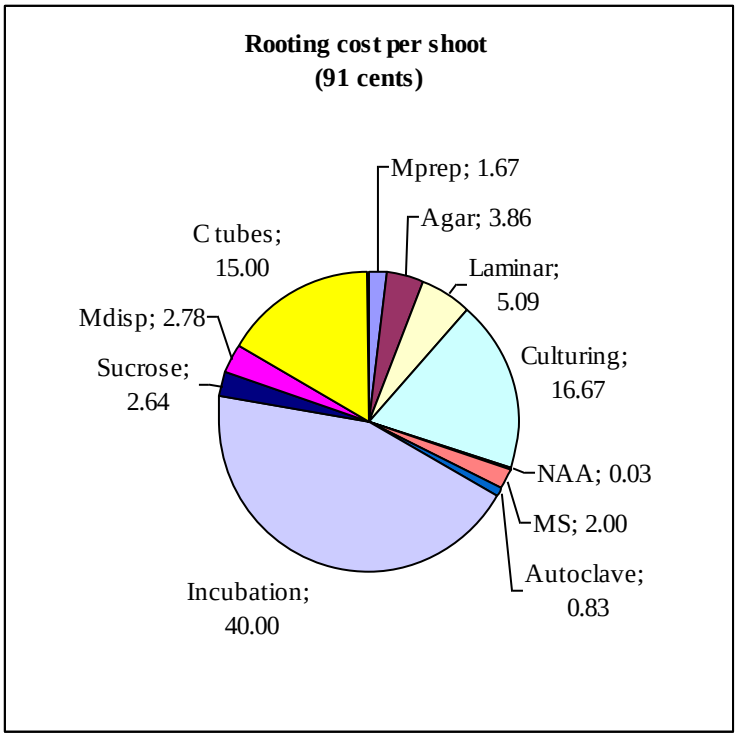


Figure (3.21). Rooting cost items in cents per shoot of pineapple rooted in culture tubes containing 10 ml of agar solidified (7 g/l) quarter strength MS medium enriched with sucrose at 30 g/l and IBA 0.5 mg/l (for Moris) and NAA at 1.0 mg/l (Smooth cayenne). The medium was autoclaved at 121 ^{0}C and 1.5 kg/cm for 25 minutes, shoots were cultured at density of one shoot per culture and incubated for 60 days under constant temperature (25 ^{0} C) and 16 hours of light provided by cool white florescent lamps.

Mprep (Medium preparation), Mdisp (Medium dispensing); Ctubes (Culture tubes).

Culturing (separation and culturing of shoots under laminar).

Autoclave, Laminar, Incubation (electricity cost of operating autoclave, laminar and incubation room)

mg/l was the best hormone for rooting of Smooth cayenne but should be used in full strength MS.

Judging by the rooting responses, our results supported the use of NAA at 1.0 in full strength medium for Smooth cayenne and IBA at 2.0 in quarter strength medium for Moris as the best compromise for highest response of all rooting aspects and demonstrated that the optimal treatment for one cultivar could be the least effective for others. However, interm of cost of rooting per shoot none of these rooting treatments could be used for commercial propagation. The cost per root using the optimal hormone treatment was 101 cents (Figure, 3.20) and using the optimum medium strength was 91 cents (Figure, 3.21). Although reducing the medium strength from full to quarter and the medium volume per culture from 20 to 10 ml (Figure, 3.20 and 3.21) reduced the cost of MS and agar by 50 % (from 16 and 7.7 cents to 2 and 4 cents per shoots) and autoclaving the medium using large glass jars and dispensing the medium into culture tubes under laminar reduced the cost of autoclaving by 88 % (from 7.5 cents to only 1 cent per shoot), the cost of rooting still very high and further work is needed to minimize the cost of rooting. Other factors beside the medium strength need to be tested for reducing the cost and maintaing the rooting quality above the minimum required for survival of acclimatization. Without developing low cost rooting treatment complains about the high cost of micropropagation will always be around even if highest multiplication of shoots at lowest cost were obtained.

3.3.3. Effect of shoot sizes and density on the *in vitro* rooting of Moris pineapple

Analysis of variance (Table, 3.38 and 3.39) indicated that shoots density per culture had no direct (main) or indirect (interaction with shoot size) effect in any of the rooting aspects. The shoot sizes also had no direct or indirect significant effect on rooting percentage, root number and length per shoot, but affected the plantlets height significantly. On average, about 82 % of the shoots developed 4 roots per shoot, each about 17.7 mm long. However,

according to height, the plantlets could be grouped into three significantly different groups. The tallest plantlets (76 mm) were obtained when 15 mm long shoots were cultured at density of 3 shoots per culture. The shortest plantlets of about 35 mm tall obtained when 5 mm long shoots were cultured at density of 1, 2 and 5 shoots per culture, 10 mm long shoots cultured at density of 2 shoots and 15 mm long shoots cultured at density of 4 shoots per culture. Increasing the density of the 5 and 15 mm long shoots from 1 to 3 shoots per culture increased the plantlet heights from 34.7 to 45.3 mm and from 48.3 to 76 mm respectively. But, the height of plantlet which developed from the 5 mm long shoots declined to 35 mm and that of the 15 mm long shoots declined to 41.7 mm as the density increased to 4 shoots per culture. The plantlets heights of the 10 and 20 mm long shoots, on the other hand, were not affected by the shoots density with an intermediate plantlet height of 65 mm tall

At density of 1 and 5 shoots per culture, the height of the plantlets developed from the 10, 15 and 20 mm long shoots was not significantly different (about 53.7 mm tall), but was 1.5 times taller than the plantlets from the 5 mm long shoots (35 mm tall). At density of 2 shoots per culture, the 5 and 10 mm long shoots resulted in shorter plantlets (about 32.5 mm tall) than that from the 15 and 20 mm long shoots (57.7 mm tall). At density of 3 and 4 shoots per culture, there were no difference between the height of the plantlets that developed from the 5, 10 and 20 mm long shoots (about 53.4 mm tall). However, plantlets developed from the 15 mm long shoots at density of 3 shoots per culture were taller (76 mm) than that developed from 15 mm long at density of 4 shoots per culture (41.7 mm) and all other shoot sizes at all shoots density. The height of the plantlets which developed from the 20 mm long shoots were not affected by the shoots density per culture (about 57.4 mm tall) and was in generally of equal height to that developed from 10 and 15 mm long shoots but longer than that developed from the 5 mm long shoots. Except when the shoot

size was 15 mm long, increasing of the shoots density per culture had no negative effect on the plantlet height. Overall sizes of the shoot used for rooting, the plantlets height (50.5 mm) were not affected by the shoots density per culture. However, overall density of shoots, the plantlets height increased from 39.2; 49.6; 55.9 to 57.4 mm as the shoots size increased from 5, 10, 15 to 20 mm in length. In summary, the plantlet height was affected mainly by the shoot sizes more than by shoots density and during rooting stage a density of 5 shoots per culture could be used for any shoots size of 5 up to 20 mm long. Estimation of cost of rooting, on the other hand, showed that the total cost at all shoots density was RM 2.92 and the cost per shoot decreased as the shoots density increased. The cost per shoot at density of 1, 2, 3, 4 and 5 shoots per culture was 97.5; 48.8; 32.5; 24.4 and 19.5 cents respectively (Figure, 3.22A). Electricity during incubation was the major cost factor of rooting (41 %) followed by labor wages (17 %) and culture tubes (15 %). MS, laminar and autoclave account for 8, 6 and 1 % of the rooting cost per shoot (Figure, 3.22B). The cost of rooting was 4 to 6 times higher than the cost of multiplication.

Shoots used in most *in vitro* rooting studies of pineapple were usually 20 mm long. However, at each multiplication cycle and of the total shoots over several consecutives cycles of multiplication, 50 % of these shoots are shorter than 20 mm in length. The high frequency of short shoots was seen as problem and elongation stage was proposed to increase the shoot length (Escalona *et al*., 1999; Firoozabady and Gutterson, 2003). Surprisingly, in spite of its important, testing the rooting ability of shoots of different size at different shoots density per culture was ignored by all researches. Neither shoot size nor the density effect on any of the rooting aspects and cost of rooting were reported. This study demonstrated that three of the rooting parameters (rooting percentage, root number and length) were not affected by the shoots size. All shoots irrespective of the shoot size

(5, 10, 15 and 20 mm) had equal rooting percentage, formed equal root number and developed roots of equal length. The only different between shoots of different size was the plantlets height. Longer shoots resulted in taller plantlets. Shoots as long as 5, 10, 15 and 20 mm were rooted and developed into 39, 50, 56 and 57 mm tall-plantlets after 60 days of incubation in simple rooting medium. The shoot density per culture, on the other hand, had no effect on all rooting aspects. Irrespective of the shoot size (5 to 20 mm long), increasing the density up to 5 shoots per culture had no negative effect on the rooting responses. It either improved or had equal effect on the plantlets height and other rooting aspects of Moris pineapple. Hence density of 5 shoots per culture is recommended. Increasing the explants density naturally will decrease vessels; medium and shelving space and consequently the cost. But it has to be sure that the rooting quality is proper for acclimatization stage. Firoozabady and Gutterson (2003) did mention that rooting were conducted using half strength liquid MS medium in a tray but did not report the number of shoots and the medium volume per the tray and the tray size.

Literatures indicated that *in vitro* obtained pineapple plantlets (rooted shoots) could easily be acclimatized. Survival rate of 95 % were obtained using 35 (Fitchet, 1990a), 50 (Ko *et al*., 2006), 70 (Vesco *et al*., 2001) and 80 mm (Be and Debergh, 2006) tall plantlets. Others (Gangopadhyay *et al*., 2005; Akbar *et al*., 2003; Soneji *et al*., 2002a; Rahman *et al*., 2001), although they did not report the plantlet height at the end of rooting stage, reported that 90 % of the plantlets survived acclimatization. Most importantly is that Moris pineapple shoots even as short as 5 mm could be rooted *in vitro* at density of 5 shoots per culture and developed into 35 mm tall plantlets taller than the plantlet height required for survival during acclimatization (35 mm tall plantlets). Instead of adopting the costly elongation stage and *ex vitro* acclimatization (Escalona *et al*., 1999; Firoozabady and

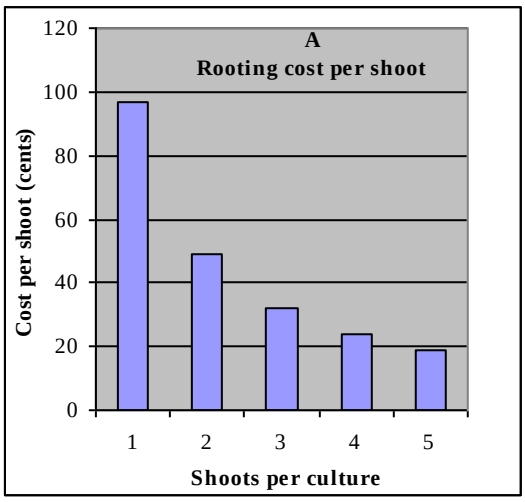
Table (3.38). Significant of main and interaction effect of shoot densities and sizes on the *in vitro* rooting of Moris pineapple

Factors	**df**	**Rooting parameters**			
		Rooting %	Root No.	Root length	Plantlet height
Shoots density	4	0.9863	0.9611	0.1328	0.4137
Shoots size	3	0.8539	0.6402	0.9826	0.0246*
Density*size	12	0.4876	0.8419	0.7856	0.5195
Error	40				
Total	60				

Table (3.39). Effect of shoot size and densities on the *in vitro* rooting of Moris pineapple

Shoots density	**Shoots size (mm)**				
	5	10	15	20	Average
Plantlet height (mm)					
1	34.7 b	57.7 ab	48.3 ab	58.7 ab	49.8
2	27.7 b	37.3 b	53.7 ab	61.7 ab	45.1
3	45.3 ab	51.7 ab	76 a	61 ab	58.5
4	53.3 ab	49 ab	41.7 b	60.3 ab	51.1
5	35 b	52.3 ab	60 ab	45.3 ab	48.2
Average	39.2 B	49.6 AB	55.9 A	57.4 A	50.5
Rooting %					
1	100	66.7	66.7	100	83.3
2	66.7	100	83.3	100	87.5
3	77.7	100	77.7	66.7	80.5
4	91.7	91.7	75	50	77.1
5	86.7	86.7	93.3	60	81.7
Average	84.53	89	79.2	75.33	82
Root No					
1	3.3	2.7	4	5.3	3.8
2	3	3.3	4	5	3.8
3	4.3	4.3	6.3	3.3	4.6
4	3	4.7	4.3	4.7	4.2
5	3.3	5	6	2.3	4.2
Average	3.4	4	4.93	4.13	4.1
Root length (mm)					
1	9.3	12.7	9.7	14	11.4 B
2	8.3	12.3	19.7	19	14.8 AB
3	20	15	23.7	17.3	19.0 AB
4	29	19.3	18.3	31.3	24.5 A
5	16	28	22.3	9.3	18.9 AB
Average	16.5	17.5	18.7	18.2	17.7

Data were mean of 9 explants per treatment cultured on 10 ml of agar solidified full strength MS medium enriched with sucrose at 30 g/l and IBA at 2.0 mg/l, adjusted to pH 5.7 and incubated for 60 days
Means of the same parameters followed by same letters were not significantly different at $p \leq 0.05$ as testd by Duncan Multiple Range Test.



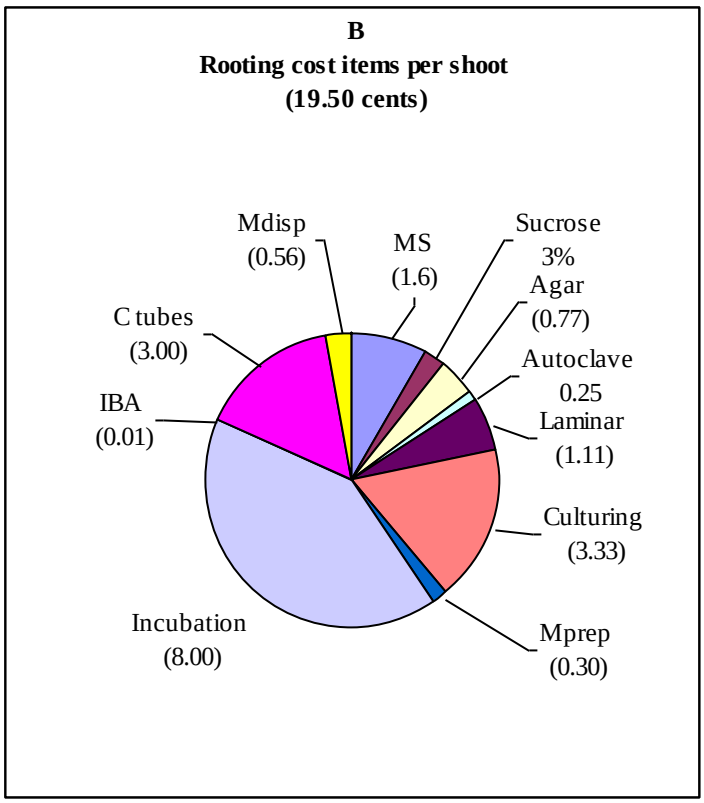


Figure (3.22). Rooting cost per shoot of Moris pineapple at different shoot density per culture (A) and cost items per shoot in cents (B). Shoots were cultured in10 ml of agar solidified (7 g/l) full strength MS enriched with sucrose **at** 30 g/l and IBA at 2.0 mg/l. Medium was autoclaved at 121^0 C and 1.5 kg/cm^2 for 25 minutes in glass jars and then dispensed into culture tubes under laminar and the culture were incubated for 60 days at constant temperature (25^0 C) and 16 hour of light provided by cool white florescent lamps.

Mprep (Medium preparation), Mdisp (Medium dispensing); Ctubes (Culture tubes).

Culturing (separation and culturing of shoots under laminar).

Autoclave, Laminar, Incubation (electricity cost of operating autoclave, laminar and incubation room)

Gutterson, 2003), the same goal could be achieved by passing the short shoots to simple and cheaper rooting medium and then to acclimatization.

Bergmann and Whetten (1998).reported that using of longer shoots for rooting of *Paulownia elongate* resulted in higher rooting percentage, more and longer roots than shorter shoots. Konan *et al*. (2007) reported that at density of 3 shoots per culture, the rooting of oil palm was under influence of coupling factors of shoot sizes. In single culture vessel, rooting of one shoot effected the rooting of the others and the best rooting obtained when the shoots in a single culture were of different sizes. When all or two of the shoots were of small size, the shoots failed to root. In this study no mixing shoot of different sizes was tested. However, at equal density, the differences in rooting response of the shoot of different sizes were not significant. The coupling effect may not be crucial for root induction and development of Moris pineapple roots. However, the possibility of coupling factor of different shoots size on the plantlets height need to be investigated. Compared to density of one shoot, Using of higher density of five shoots per culture reduced the rooting cost from 97 cents at density of one shoot per culture to 19.5 cents (Figure, 3.22A). Yet, the cost is still above commercial level of mass propagation. Figure, 3.22B showed that 15 % of the rooting cost was due to medium (MS, agar and sucrose). Shoot density higher than 5 shoots per culture were not tested, but, it seemed possible that the cost of rooting stage could be reduced much more by using of liquid medium at lower strength in larger vessel and higher shoots density and lower medium volume per culture.

3.3.4. Effect of pH, sucrose concentrations and medium states on the *in vitro* rooting of Moris pineapple

Analysis of variance (Table, 3.40) showed that the initial pH value of the medium did not have a significant direct (independent) effect on any of the rooting parameters and did not interact significantly with agar concentrations (medium state). However, the pH did have indirect significant effect via interaction with sucrose on all aspects of rooting and via interaction with both of sucrose and agar (medium states) on all rooting aspects except the plantlets height. Both of sucrose and agar had a significant direct (independent) effect on all rooting parameters and interacted significantly (indirect effect) with each other. However, while the agar did not interact with the medium pH, the sucrose interacted significantly with the medium pH and all of the three factors had significant interaction on all rooting aspects except plantlets height. Table 3.41 showed that overall pH and sucrose concentrations, liquid medium resulted in taller plantlets (51.06 mm), more (5.38 roots) and longer roots (16.31 mm) than solid medium (39 mm tall plantlets, 3.13 roots per shoots, 8.9 mm long each) but the rooting percentage of both liquid and solid medium were not significantly different (about 62 %). Similar, overall medium states and pH values, the largest number (5.4 roots), longer roots (16.25 mm) and highest rooting percentage (76.3 %) obtained in medium enriched with sucrose at 20 and 30 g/l. However, sucrose at 20 g/l produced the tallest plantlets (52.8 mm) and sucrose at 30 g/l resulted in an intermediate plant height (48.08 mm). Sucrose at 10 and 40 g/l resulted in the lowest rooting percentage (47.0), fewest (2.1 roots) and shortest (6.7 mm long) root, but the plantlets on medium containing sucrose at 10 g/l was taller (42.33 mm) than that in medium with 40 g/l (31.9 mm). Overall medium states and sucrose, the pH values of the medium had no significant effect on any of the rooting responses (Table, 3.41).

All shoots (100 %) could be rooted in liquid media enriched with sucrose at 10 g/l and pH 6.0; sucrose at 20 g/l and pH 5.0 and 6.5; sucrose at 30 g/l and pH 5.0 and on semi solid media enriched with sucrose at 20 g/l and pH 6.5; sucrose at 30 g/l and pH 5.0 and sucrose at 40 g/l and pH 6.5 (Table, 3.41). The highest rooting percentage in solid media, on the other hand, was 89 %, obtained in medium enriched with sucrose at 20 g/l and pH 5.0 and sucrose at 30 g/l and pH 5.0 and 5.5. Rooting percentage as low as 22 % obtained in liquid and solid medium enriched with sucrose at 10 g/l and pH 5.5 and as low as 11 % obtained in semi solid medium enriched with sucrose at 40 g/l and pH 5.0 and 6.0. Overall pH, the highest rooting percentage (88 %) obtained on liquid medium enriched with sucrose at 30 g/l while the lowest (31.3 %) obtained on semi solid medium enriched with sucrose at 10 g/l. Seven different combinations of media state, sucrose concentrations and pH values none of them included solid media and pH 5.5 resulted in 100 % rooting of shoots. Selection among these combinations would depend on how these combinations affected the other rooting aspects.

The highest root formation (10 to 11 roots per shoot) occurred on liquid medium enriched with 20 and 30 g/l and pH 5.0 and 5.5 and solid medium enriched with sucrose at 30 g/l and pH 5.0. The lowest root formation (1 root) occurred in almost all (75 %) of the combinations in which the sucrose enrichment was 10 g/l and in 50 % of the combinations in which sucrose enrichment was 40 g/l. Overall pH, the highest root formation (8.8 roots per shoot) obtained in liquid medium enriched with sucrose at 30 g/l and the lowest (1 root/shoot) on semi solid medium enriched with sucrose at 10 g/l. Assessment based on root number did not distinguished between solid and liquid states of the medium. However, it limited the sucrose choice between 20 and 30 g/l. On the other hand, the longest root (39 mm) obtained on liquid medium enriched with sucrose at 30 g/l and pH 5.0 and the shortest (3.0 mm) on semi solid medium enriched with sucrose at 10 g/l and pH 6.5;

sucrose at 40 g/l and pH 5.0 and 5.5 and solid medium enriched with sucrose at 10 g/l and pH 5.5. Overall pH, the longest roots (29 mm) obtained in liquid medium enriched with sucrose at 30 g/l and the shortest (5.3 mm) on semi solid enriched with sucrose at 10 g/l. A medium which was optimal for all of the three rooting parameters (rooting percentage, root number and length) is liquid medium enriched with sucrose at 30 g/l and adjusted to pH 5.0. However, if the root length ignored, the sucrose could be reduced to 20 g/l.

The tallest plantlet (71 mm) obtained in liquid medium enriched with sucrose at 20 g /l and adjusted to pH 6.0 and the most stunted plantlets (15.0 mm) obtained on semi solid enriched with sucrose at 40 g/l and adjusted also to pH 5.0 and 6.0. Overall pH values, liquid medium enriched with sucrose at 20 g/l resulted in the tallest plantlets (63 mm) and liquid and semi solid medium enriched with sucrose at 40 g/l resulted in the shortest plantlets (30.25 and 28.25 mm respectively). No significant different on height between plantlets on solid medium enriched with sucrose at 10 and 40 /l and semi solid enriched with sucrose at 10 g/l. Plantlet height supported selection of liquid medium as other parameters did, but at different concentration of sucrose (20 g/l) and pH value (6.0).

According to plantlets height, liquid medium enriched with sucrose at 10 and pH 6.0; sucrose at 20 g/l and pH 5.0 and 6.0; sucrose at 30 g/l and pH 5.0 and semi solid medium enriched with sucrose at 20 g/l and pH 6.0 were the best treatments. Any of these treatments resulted in 66 to 71 mm tall plantlets. The rooting percentage supported use of liquid medium enriched with sucrose at 10 g/l and pH 6.0; sucrose at 20 g/l and pH 6.5; sucrose at 30 g/l and pH 5.0 (all shoots rooted). The other best treatments for plantlet height failed to induce more than 55 % rooting. According to the highest value of three parameters (plantlet height, rooting percentage and root per shoot), liquid media enriched with sucrose at 20 and 30 g/l and adjusted to pH 5.0 were the best combinations. However, the root length of the second combination was two times longer than the first and was the

longest of all treatments. Generally, for all rooting parameters, liquid medium resulted in better response than solid and semi solid medium. Of all of the 48 combinations, 5 resulted in tallest plantlets (4 liquid, 1 semi solid), 7 in 100 % rooting (4 liquid, 3 semi solid), 5 in highest root formation (4 liquid, 1 solid) and 1 combination in the longest roots (in liquid). The choice between enrichment with 10 and 20 grams of sucrose would depend on the rooting parameter of particular interest and pH of the medium. Physiologically, the highest and the fastest responses, the best is the treatment. However, in practice, selection of the best treatment depends on management and cost assessment. Longer and more roots would create handling problem during hardening and higher sucrose increased the cost of production. Since survival and growth rate during acclimatization depends mainly on plantlets height, liquid medium enriched with sucrose at 10 g/l and adjusted to pH 6.0 would be the lowest cost rooting treatment. Estimation of cost showed that rooting cost per shoot was 29.5 cents mostly due to cost of electricity of incubation room, labor and culture tubes (Table 3.42; Figure, 3.23).

Enrichment of the rooting medium with sucrose at 30 g/l and adjusting the pH to 5.7 were originally reported using solid medium (Mathew *et al*., 1976) and still being used 30 year after, Nevertheless, no supporting data for using of this particular pH value and sucrose concentration was provided. Soneji *et al.* (2002a) demonstrated that good rooting could be obtained in liquid filter paper bridged medium enriched with sucrose at 20 and even at10 g/l. However, they did not compare the results with sucrose at 30 g/l, different pH values and agar solidified medium and did not report the plantlets height, the most important quality of rooted shoots. The results (Table, 3.41) showed that in solid medim, all rooting parameters in media enriched with sucrose at 30 g/l and pH 5.5 were better than any other pH adjustments and sucrose concentrations. At fixed sucrose of 30 g/l and pH of 5.5, liquid and solid media resulted in almost equal plantlet height and rooting percentage.

However, the liquid medium was super than solid medium for root number and length. This study indicated that 100 % rooting and 66 to 71 mm tall Moris plantlets could be obtained in medium enriched with 10, 20 and 30 g/ provided the medium adjusted to proper pH. However, using of sucrose at 30 g/l resulted in more and longer roots than the other sucrose concentration and would create problem during transfer the plantlets to acclimatization stage.

It is clear that arbitrary selection and fixing sucrose at 30 g/l and pH at 5.7 and limiting the comparison to one rooting parameter (the commonly used practice) optimization of rooting would not be achieved. Comparison of all combinations of sucrose, pH and medium states indicated that the agar could be completely eliminated and the sucrose could be lowered to 10 g/l provided that the medium pH adjusted to proper value (Table, 3.41). At any of the 48 combinations of medium state, sucrose and pH, except when sucrose was at 40 g/l, the highest rooting percentage and tallest plantlets obtained in liquid medium. Liquid medium, except in 3 combinations, resulted also in more and longer roots than solid and semi solid medium. Successful rooting of pineapple in liquid medium was reported before but the shoots were supported by sponge matrix (Gangopadahyay *et al*., 2005) and filter paper bridge (Soneji *et al*., 2002a; Mathew and Rangan, 1981; 1979) rather than direct placement of shoots on static liquid as done in this study. In addition, the sucrose and pH were fixed at one level and the plantlets height, an important shoot quality, was not reported. Rooting of all Moris shoots (100 %) could be obtained in liquid medium if the sucrose kept constant at 30 g/l and the pH lowered to 5.0, both lowered, sucrose to 20 g/l and pH to 5.0; sucrose lowered to 20 and 10 g/l and pH increased to 6.0 and if both increased, sucrose to 40 g/l and pH to 6.0. In liquid medium lowerin both, sucrose to 20 and pH to 5.0, lowering sucrose to 20 but keeping pH at 5.5 produced equal number of root as that on sucrose at 30 g/l and pH 5.5. Compared to sucrose at 30 g/l and pH 5.5

maintaing the sucrose at 30 g/l but lowering the pH to 5.0 instead of 5.5, almost doubled the root number and roots length of the shoot rooted in liquid medium and tripled that of the shoots rooted in semi solid medium. Similar, lowering the sucrose to 10 g/l and increasing the pH of liquid medium to 6.0 resulted in taller plantlets while lowering sucrose to 20 g/l and increasing pH to 6.0 in semi solid resulted in shorter plantlets.

Compared to solid medium enriched with sucrose at 30 and pH 5.5, the plantlet height could be 30 % taller by using liquid medium, lowering the sucrose to 10 g/l and increasing the medium pH to 6.0. The roots per shoot could be 30 % more by lowering the sucrose to 20 and pH to 5.0. If the plantlet height was not used as parameter for assessment and pH was fixed at 5.7, as it usually done in all rooting studies of pineapple, sucrose at 10 g/l, the cheapest treatment of all would be immediately rejected. Simple manipulation of the medium pH reduced the sucrose concentration by two times while maintaining equal rooting percentage and production of taller plantlets. Using of cheap sucrose alternative was suggested as mean of cost reduction in micropropagation of banana (Kodym and Zapata, 2001) and several plant species (Gangopadhyay *et al*., 2002). However, using of liquid medium and adjusting to specific pHvalue might be even better method for reduction the sucrose concentration and other sucrose alternatives to minimal valueand consequently the cost of medium during rooting. Obtaining of 70 mm-tall Moris plantlets in liquid medium enriched with sucrose at 10 g/l by adjusting of the initial pH to 6.0 instead of 5.5 is an important and simple step not only for reduction of sucrose cost but most importantly for plantlet elongation.

The shoots rooting response to the sucrose concentration depended on the medium state and pH adjustment. At different pH and medium state, there was different concentration range of sucrose for optimal rooting. In all medium states (liquid, semi solid and solid) in which the pH was adjusted to 5.0 and 5.5, the plantlet height, rooting

percentage, root number and length increased as the sucrose increased up to 30 g/l. However, if the medium pH was adjusted to 6.0, the decline in the rooting responses in solid and semi solid started after the sucrose concentration reached 20 g/l while in liquid medium the decline started after 10 g/l. If the medium pH adjusted to 6.5, the decline in the rooting response in liquid and semi solid started after the sucrose concentration reached 20 g/l while in solid medium started after 10 g/l. In other words, the sucrose had inhibitory and promotion effect that could be manipulated by medium state and pH adjustment. Using different plants, other researchers had noticed the important of medium pH for rooting. Lowering pH from 5.7 to 4.7 reduced the rooting percentage of Geraldton wax from 63 to 20 % (Page and Visser, 1989) while lowering the pH from 5.7 to 4.0 increased the rooting percentage of Australian woody plants from 28 to 100 % (William *et al*., 1995). In solid WPM enriched with sucrose at 30 g/l, lowering the pH from 5.7 to 3.5 decreased the rooting of *Choisya ternata* by up to 60 % and *Delphinium* by 15 % (Leifert *et al*., 1992). .At fixed concentration of sucrose (30 g/l), using of solid and liquid MS medium and adjusting the pH to (4.2, 4.7, 5.2, 5.7, 6.2) did not effect the rooting percentage of *Maranta leuconeura* cv Kerchoviana (Ebrahim *et al*., 2002). However, Bennett *et al* (2004) reported that the lower rooting percentage (62 %) and few roots (4 roots) of *Eucalyptus glabulus* were mainly due to presence of NH_4NO_3. In medium devoid of NH_4NO_3, rooting percentage of 94 % and 7 roots per shoots was obtained over pH range of 4.0 to 6.0.

Table. (3.40). Significant of main and interaction effect of medium states, sucrose concentrations and initial pH values of full strength MS medium enriched with IBA at 0.5 mg/l on the on *in vitro* rooting of Moris pineapple

Factors	**df**	**Rooting parameters**			
		Plantlet height	Rooting %	Root No.	Root length
Medium states	2	0.0001**	0.1310	9.09E-07 **	5.1E-05 **
Sucrose concentrations	3	5.1E-08 **	0.0001 **	1.5E-12 **	7.5E-09 **
pH values	3	0.8404	0.5973	0.4395	0.1909
State*Sucrose	6	0.0103 *	0.4861	0.0055 **	0.1382
State*pH	6	0.5682	0.2698	0.2666	0.4854
Sucrose*pH	9	1.07E-05 **	0.0238 *	1.2E-06 **	0.0004 **
State*Sucrose*pH	18	0.2315	0.0085	2.3E-05 **	0.0038 **
Error	96				
Total					

Table (3.41). Effect of medium states, sucrose concentrations and initial pH values of full strength MS medium enriched with IBA at 0.5 mg/l on the in vitro rooting of Moris pineapple

States	**pH**	**Sucrose (g/l)**			
(Agar/l)		10	20	30	40
Plantlet height (mm)					
Liquid	5	55 abcdefghi	56 abcdefgh	66 abc	22 klm
(0.0 g/l)	5.5	61 abcde	57 abcdefg	60 abcdef	22 klm
	6	70 ab	71 a	56 abcdefgh	31 ghijklm
	6.5	38 cdefghijklm	68 ab	38 cdefghijklm	46 abcdefghij
		56 AB	63 A	55 AB	30.2 D
Semi solid	5	52 abcdefghij	53 abcdefghi	56 abcdefgh	16 lm
(3.5 g/l)	5.5	32 fghijklm	42 bcdefghijklm	55 abcdefghi	24 jklm
	6	38 cdefghijklm	65 abcd	48 abcdefghij	15 m
	6.5	27 ijklm	52 abcdefghij	27 ijklm	58 abcdefg
		37.2 CD	53 AB	46.5 BC	28.2 D
Solid	5	33 efghijklm	46 abcdefghij	51 abcdefghij	32 fghijklm
(7 g/l)	5.5	28 hijklm	42 bcdefghijklm	**56 abcdefgh**	27 ijklm
	6	30 ghijklm	43 abcdefghijklm	27 ijklm	42 bcdefghijklm
	6.5	44 abcdefghijk	39 cdefghijklm	37 defghijklm	48 abcdefghij
		33.75 CD	42.5 BCD	42.75 BCD	37.25 CD
Rooting %					
Liquid	5	44.3 abcde	100 a	100 a	33.3 abcde
	5.5	22.3 de	89 abc	96.3 ab	37 abcde
	6	100 a	44.3 abcde	77.7 abcd	78 abcd
	6.5	33.3 abcde	100 a	78 abcd	78 abcd
		49.98	83.08	88 a	56.58 abcd
Semi solid	5	66.7 abcde	77.7 abcd	100 a	11 e
	5.5	22 bcde	55.7 abcde	55.7 abcde	44.3 abcde
	6	22 bcde	89 abc	77.7 abcd	11 e
	6.5	14.7 de	100 a	55.7 abcde	100 a
		31.3 d	80.6 ab	72.3 abc	41.6 cd
Solid	5	33.3 abcde	89 abc	89 abc	44.3 abcde
	5.5	22 bcde	77.7 abcd	89 abc	55.3 abcde
	6	44.3 abcde	77.7 abcd	44.3 abcde	89 abc
	6.5	78 abcd	33.3 cde	33.3 cde	44.3 abcde
		44.4 cd	69.4 abc	63.9 abcd	58.3 abcd

Liquid (0.0 agar), Semi solid (3.5 g /l) and Solid (7.0 g/l) full strength MS medium enriched with IBA at 0.5 mg/l.
Data were means of 9 shoots after 60 day of incubation.
Means of each rooting parameter followed by same letters were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range Test.

Table (3.41). Continued.

States (Agar/l)	**pH**	10	**Sucrose (g/l)** 20	30	40
Root per shoot					
Liquid	5	1 hij	10 ab	11 a	1 hij
(0.0 g/l)	5.5	1 hij	10 ab	11 a	3 defghij
	6	5 abcdefg	3 defghij	7 abcde	4 cdefghi
	6.5	1 hij	6 abcdef	6 abcdef	6 abcdef
		2 CDE	7.3 AB	8.8 A	3.5 CD
Semi solid	5	1 hij	3 defghij	8 abc	0 j
(3.5 g/l)	5.5	1 hij	2 fghij	3 defghij	2 fghij
	6	0 j	7 abcd	6 abcdef	1 ij
	6.5	0 j	5 abcdefgh	2 efghij	4 cdefghi
		1 E	4.3 CD	4.8 BC	1.8 DE
Solid	5	1 hij	5 bcdefghi	11 a	1 hij
(7 g/l)	5.5	1 hij	2 efghij	7 abcd	4 cdefghi
	6	2 efghij	2 efghij	1 hij	6 abcdef
	6.5	4 cdefghi	1 ij	1 ij	1 hij
		2 CDE	2.5 CDE	5 BC	3 CD
Root length (mm)					
Liquid	5	12 cdefghi	22 abcde	37 a	6 fghi
	5.5	7 ghi	25 abcd	26 abc	4 ghi
	6	14 abcdefgh	10 cdefghi	35 ab	10 bcdefghi
	6.5	2 hi	18 abcdefg	10 bcdefghi	23 abcde
		8.8 CD	18.5 AB	27 A	10.8 BCD
Semi solid	5	7 defghi	12 bcdefghi	22 abcde	1 i
	5.5	2 hi	9 cdefghi	6 fghi	5 fghi
	6	3 hi	23 abcde	12 bcdefghi	1 i
	6.5	1 i	23 abcde	6 efghi	13 bcdefgh
		3.3 D	16.75 AB	11.5 BC	5 CD
Solid	5	8 efghi	13 bcdefghi	24 abcd	4 ghi
	5.5	1 i	12 bcdefghi	22 abcdef	5 ghi
	6	5 fghi	9 cdefghi	4 ghi	12 bcdefghi
	6.5	10 cdefghi	5 ghi	5 ghi	4 ghi
		6 CD	9.8 BCD	13.75 BC	6.3 CD

Liquid (0.0 agar), Semi solid (3.5 g /l) and Solid (7.0 g/l) full strength MS medium enriched with IBA at 0.5 mg/l.
Data were means of 9 shoots after 60 day of incubation.
Means of each rooting parameter followed by same letters were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range Test.

Table (3.42). Estimated rooting cost per shoot of Moris pineapple at different medium states and sucrose concentration.

Medium states (agar g/l)	Sucrose concentration (g/l)				
	10	20	30	40	Average
	Cost per shoot (cents)				
Liquid (0.0)	29.5	29.8	30.0	30.3	29.9
Semi solid (3.5)	30.1	30.3	30.5	30.8	30.4
Solid (7.0)	30.6	30.8	31.0	31.3	30.9
Average	30.1	30.3	30.5	30.8	30.4

Average of 36 shoots rooted in full strength MS medium enriched with IBA at 0.5 mg/l. Media were autoclaved at 121 ^{0} C and 1.5 kg/ cm ^{2} for 25 minutes in glass jars and then dispensed into cultures tubes (10 ml per tube) under laminar. Three shoots were cultured per tube and incubated for 60 days under constant temperature 25^{0} C and 16 hour of light provided by cool white florescence lamps.

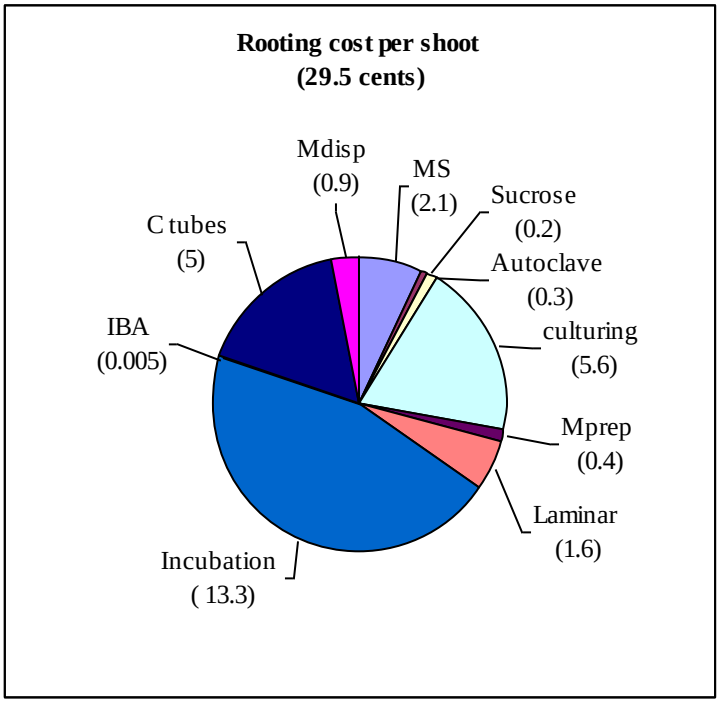


Figure (3.23). Estimated cost item per shoot of Moris pineapple.
Shoots rooted in 10 ml of liquid full strength MS medium enriched with IBA at 0.5 mg/l. Media were autoclaved at 121 ^{0} C and 1.5 kg/ cm ^{2} for 25 minutes in glass jars and then dispensed into cultures tubes (10 ml per tube) under laminar. Three shoots were cultured per culture tube and incubated for 60 days under constant temperature 25 ^{0} C and 16 hour of light provided by cool ehite florescence lamps.
Mprep (Medium preparation); Mdisp (Medium dispensing); Ctubes (Culture tubes).
Culturing (separation and culturing of shoots under laminar)
Autoclave, Laminar, Incubation (electricity cost for operating of autoclave, laminar and incubation room).

Harbage *et al*. (1993) noticed that the optimal pH for root formation of Gala apple varied at different concentrations of IBA. Increasing the sucrose concentration shifted the *in vitro* rooting dose-response curve of Jork 9 apple to auxin to the right (Calamar and de Klerk, 2002). Our results indicated that for *in vitro* rooting of Moris pineapple, the rooting dose response curve to sucrose could be shifted by medium pH and the sucrose concentration could be minimized by adjusting the medium to proper pH. Compared to shoots rooted in agar solified, using of liquid medium reduced the cost only by 1 cent per shoots and compared to sucrose at 40 g/l, using of 10 g/l sucrose reduced the cost by 1 cent per shoot (Table, 3 42). For rooting cost reduction the focus should be in shortening incubation and using of large vessel and high explant density while maintaining the root quality above the minimum required for survival of acclimatization.

3.3.5. Effect of sucrose concentrations and incubation periods on the *in vitro* rooting of Moris pineapple

Two ways analysis of variance (Table, 3.43) showed that sucrose and incubation periods had significant effect on all *in vitro* rooting responses of pineapple. However, the sucrose seemed to have higher influence on rooting percentage, root number and length than the incubation period while incubation affected plantlets height more than the sucrose. The two factors exerted their influence independently from each other except in root length where a significant interaction was detected at $p < 0.055$. Overall sucrose, the rooting response after 30 and 45 days incubation were not significantly different and resulted in the lowest responses of all rooting aspects. About 37 % of the shoots rooted, forming 2 roots per shoot each 4 mm long and 24 mm tall plantlets. Intermediate responses (56 5 % rooting, 2 roots/ shoots, 7 mm long roots and 32 mm tall plantlets) obtained after 60 days of incubation. Incubation for 75 days, resulted in the highest rooting percentage (76.4 %), roots per shoot (3 roots), longest roots (11 mm long) and tallest plantlets (39.17 mm tall).

Similar, overall incubation periods, none of the shoots cultured on MS supplemented with sucrose at 5 g/l produced roots and the plantlets was the shortest (20.1 mm tall) of all sucrose concentrations. Increasing the sucrose to 10 g/l resulted in 35.4 % rooting, with about 2 roots per shoot each 6 mm long and plantlets 1.5 times taller (30.1 mm) than that in medium enriched with sucrose at 5 g/l. Compared to sucrose at 10 g/l, increasing the sucrose level to 15 grams did not improve the plantlets height (33.3 mm) and root length (8.3 mm), but doubled rooting percentage (72.4 %) and root number (3.8). Compared to sucrose at 15 g/l, increasing the sucrose up to 30 g/l did not improve plantlets height and rooting percentage, however, it decreased the root number down to 2 roots per shoot and root length to 5.3 mm.

Comparison of means indicated that at each incubation period there were different optimal levels of sucrose for each rooting parameter (Table, 3.44). The plantlet height in medium enriched with sucrose at 5 g/l after 30 days was 15.7 mm, increased to 19, 21 and 24 mm as the incubation increased to 45, 60 and 75 days respectively. About 3 mm increase every 15 days. Keeping the incubation fixed at 30 days and increasing the sucrose up to 30 g /l did not bring about any significant affect in the plantlets height. However, the responsiveness of the shoots to sucrose supplement improved if the incubation was extended for more than 30 days. Under 45 days of incubation, the plantlet height increased from 19 mm at 5 grams of sucrose to 28.0 mm and 32.3 mm as the sucrose increased to 10 and 15 g/l. Sucrose enrichment higher than 15 g/l suppressed the shoots elongation and the plantlet height decreased to 19.7 mm in medium enriched with sucrose at 25 g/l. However, the suppression effect of the high sucrose concentration could be avoided by extending the incubation for another 15 and 30 days. Under the 60 and 75 days incubation, the plantlets height increased from 21.3 and 24.3 mm at 5 grams of sucrose to reach respectively a

maximum of 36.7 at 20 grams and 46.3 mm at 25 grams of sucrose and declined afterwards to 32.7 and 42.3 mm at 30 grams of sucrose.

The highest root formation (4.7 roots / shoot) and elongation (27.3 mm) of all treatments obtained in medium enriched with sucrose at 20 g/l and incubated for 75 days. Increasing the sucrose 30 g/l decreased the root formation to 3 roots per shoot and the root length to 11 mm long. The best sucrose concentration for highest root number and longest root for the shoots incubated for 30, 45 and 60 days was 15 g/l. Under incubation regime of 30 days, the shoots developed 3 roots each 5.7 mm long while under 45 and 60 days regime the root average per shoot was 3.7 and 4.0 roots each 7.0 and 10.3 mm long respectively. Sucrose concentrations higher than 15 grams per liter had no effect on root length but the root number declined to 2 roots per shoot. On the other hand, all shoots in medium enriched with sucrose at 5 g/l failed to root even if the incubation extended to 75 days and the fewest number (one roots per shoot) and the shortest roots (1.7 mm) of all treatments obtained in medium enriched with sucrose at 10 g/l and incubated for 45 days. The highest rooting percentage after 30, 45 and 60 days of incubation were 66, 75 and 90 % obtained respectively in medium enriched with sucrose at 25, 15 and 20 g/l and declined to 33, 50 and 66 % as the sucrose increased to 30 g/l. Under 75 days, almost all shoots rooted (91.7 - 100 %) at wider sucrose range of 10 to 25 g/l. The rooting percentage declined to 75 % as the sucrose increased to 30 g/l and none of the shoots rooted in medium enriched with sucrose at 5 g/l.

Irrespective of the incubation periods, the shoots failed to form roots if the medium was supplemented with sucrose at 5 g/l. The plantlet height in the medium enriched with sucrose at 5 g/l was the shortest and the growth rate was the slowest at any incubation period. In medium enriched with sucrose at 10 g/l, the tallest plantlets (34.0 mm) obtained after 60 days incubation. Extending the incubation to 75 days did not increase the height.

The tallest plantlet in the other sucrose concentration obtained after 75 days of incubation. The response to sucrose at 15 and 20 g/l in term of plantlet height was identical and was at each incubation period the tallest than other sucrose concentrations (Table, 3.44).However, while sucrose at 15 g/l resulted in higher rooting percentage and more roots than that at 20 g/l after 30 and 45 days, sucrose at 20 g/l resulted in higher rooting percentage than that at 15 g/l when the incubation extended to 60 and 75 days but equal roots per shoot. Similar, while medium enriched with sucrose at 15 and 20 g/l resulted in equal roots length after 30 and 60 days, the root length after 45 days in medium enriched with 15 g/l was two time longer than that in medium enriched with sucrose at 20 g/l while after 75 days the root length in medium enriched with 20 g/l was three times longer than that in medium enriched with sucrose at 15 g/l. Judging by the highest value of the rooting parameters, incubation for 75 days was the best incubation period. However, each parameter had different optimal sucrose concentration. The tallest plantlets (46.3 mm) obtained in medium enriched with sucrose at 25 g/l, the highest rooting percentage (100 %) in medium enriched with sucrose at 10 and 20 g/l, while the highest average of number (4.7 roots) and longest length (27.3 mm) of roots in medium enriched with sucrose at 20 g/l. Compared to sucrose at 30 g/l and incubation for 30 days, the amount of sucrose could be reduced to 15 and 10 g/l but the incubation period need to be extended for more 15 and 45 days respectively

At any sucrose concentration, extending the incubation period improved the rooting percentage. Yet different sucrose concentrations seemed to have different effect on the root induction. In medium enriched with sucrose at 5 g/l, the shoots failed to root even if the incubation extended to 75 days. In medium enriched with sucrose at 10 g/l, no rooting occurred in the first 30 days and only 25 % rooted after 60 days, but all of the shoots rooted when the incubation extended to 75 days. That is 75 % of the rooting occurred within the last 15 days of incubation. On contrary, in medium enriched with sucrose at 15

g/l, 75 % of rooting occurred within the first 45 days of incubation and 25 % of rooting on the period between 45 and 75 days of incubation. In medium enriched with sucrose at 20 and 25 g/l, 92 and 75 % of shoots rooted within the first 60 days and 10 to 25 % rooted in the period between 60 and 75 days of incubation. In medium enriched with sucrose at 30 g/l, 25 % of the shoots failed to root even if the incubation extended to 75 days. Similar, in media enriched with 15 and 20 g/l, extending the incubation from 45 to 75 days resulted in 11 mm increase in plantlet height while in media enriched with sucrose at 25 and 30 g/l, extending the incubation from 45 to 75 days resulted in 27 and 20 mm increase in plantlet height respectively (two to three times faster growth).

Both of sucrose concentrations and incubation periods are very important for improving the rooting responses, development of good quality plantlets and for total cost assessment. However, while low sucrose reduces the sucrose total cost but long incubation increases the monthly electricity bill. To reduce the cost of sucrose and incubation, selection of the incubation period as well as sucrose should not only be base on statistical analysis of the responses but also on a compromise between the lowest possible sucrose, shortest incubation, best rooting quality and highest survival of plantlets during acclimatization. The rooting cost per shoot, on the other hand, was mainly affected by the incubation periods more than concentration of sucrose. At density of 5 shoots per culture, the minimum cost per shoot was 13 cents obtained and incubation for 30 days while the highest cost was 19 cents and incubation for 75 days irrespective of the sucrose content of the medium (Table, 3.45). The different in cost per shoot at different sucrose concentration was negligible (less than 0.1 cents) while shorter incubation of 30 days decreased the cost by 6 cents compared to 75 days of incubation. The major cause of rooting cost was incubation ((10 cents, 52 %) followed by labor wages for culturing (3.3 cents, 17 %) and

cost of culture tubes (3 cents, 16 %). The cost of sucrose on the other hand, was only 1% (0.2 cents per shoot).

Although sucrose at 20 and even 10 g/l in a liquid MS medium and 70 days of incubation resulted in an excellent *in vitro* rooting responses of Queen pineapple (Soneji *et al*., 2002a), enrichment of medium with sucrose at 30 g/l and incubation for 30 days still the most common practice for *in vitro* rooting of pineapples. In agreement with Soneji and co researchers findings, Table 3.46 shows that only one to two third of this amount of sucrose is needed to obtain the best result of all rooting parameters of Moris pineapple cultivar but the incubation should extend to 75 days. It is clear that for any rooting aspect, the commonly used sucrose enrichment (30 g/l) and incubation period (30 days) was not proper for rooting of Moris pineapples. Only 33 % of the Moris shoots rooted and the plantlets were shorter than 25 mm. In fact, medium enriched with sucrose at 30 g/l and incubated for 30 days resulted in lower rooting percentage in 16 combinations, shorter plantlets shorter in 13 combinations, fewer and shorter roots in 9 out of 24 combinations of sucrose concentration and incubation periods. At any fixed incubation period, other sucrose concentration resulted in better rooting response than sucrose at 30 g/l and at any fixed sucrose concentration, incubation longer than 30 days resulted in better rooting response of Moris pineapple. The tallest plantlets obtained in medium enriched with sucrose at 25 g/l and the highest and longest roots obtained in medium enriched with sucrose at 20 g/l and incubated for 75 days. All shoots (100%) rooted in medium enriched with sucrose at 10 and 20 g/l and incubated for 75 days.

Different rooting parameter had different optimal combination of sucrose concentration and incubation periods and none of the combinations was best by all rooting parameter. Hence the best combination depended on the parameter that serve the final goal of rooting stage and reduce the cost of rooting. For propagator, the goal is plantlets survival of

acclimatization, however, if the rooting was done for bioproduct extraction the goal would be highest percentage of rooting and formation of more and longer roots. Although statistically significant, the different in root length in medium enriched with sucrose at 15, 25 and 30 g/l over the different incubation periods was less than 5.0 mm and practically negligible. Over 70 % of the increase in root length in medium enriched with sucrose at 10 (4.3 to 16.0 mm) and 20 g/l (9.3 to 27.3 mm) occurred specifically in the period between 60 and 75 days of incubation. Hence judging by the root length, the incubation of the medium enriched with 15, 25 and 30 g/l could be terminated after 30 days while for medium enriched with 10 and 20 g/l should be extended to 75 days. Similar, the highest root number in medium enriched with sucrose at 15 (4.0 roots) and 25 g/l (3 roots) obtained after 60 days. Extending the incubation to 75 days caused no significant increase in root number. The maximum roots per shoot in medium enriched with 10 (4.0 roots) and 20 g/l (4.7 roots) obtained after 75 days of incubation. However, although statistically significant, the different in roots number between the 60 and 75 days of incubation in medium enriched with sucrose at 20 g/l was only one root while in medium enriched with sucrose at 10 g/l reached 3 roots. Hence judging by root number, sucrose at 10 g/l and 75 days and sucrose at 20 g/l and 60 days incubation could be recommended as the best compromise for lowest sucrose and shortest incubation and highest shoot formation. No root formation occurred in medium enriched with 5 g/l even if the incubation increased to 75 days. However, over 90 % of the shoots on medium enriched with 10 and 20 g/l rooted after 75 and 60 days of incubation respectively. If the rooting percentage is not important for plantlets survival during hardening, sucrose at 5 g/l and 30 days of incubation would be recommended as the least costly rooting treatment.

Dewald *et al*. (1988), Vesco *et al*. (2001), Soneji *et al*. (2002a), Ko *et al*. (2006) and Be and Debergh (2006), on the other hand, respectively reported that over 90 % of 35, 50, 60,

70 and 80 mm tall pineapple plantlets (rooted shoots) survived acclimatization stage. Escalona *et al* (1999) demonstrated that rootless shoots could be successfully hardened and *ex vitro* acclimatized provided that the shoots were 80 mm or longer. None of the rootless and even the rooted shoots (plantlets) obtained in this study was longer than 60 mm.(Table, 3. 44). All of the plantlets after 30 days of incubation irrespective of sucrose, after 45 days in medium enriched with sucrose at 10, 25 and 30 g/l and in medium enriched with sucrose at 5 g/l irrespective of incubation were shorter than 35 mm while incubation for 45 days in medium enriched with sucrose at 15 and 20 g/l and incubation for 60 days and longer irrespective of sucrose concentrations resulted in plantlets taller than 35 mm. Of the 12 combinations in which plantlets were taller than 35 mm, sucrose at 15 g/l and incubation for 45 days was the lowest sucrose and shortest incubation period. However, the root number and length and rooting percentage of the shoots incubated for 45 days was less than that obtained after 60 and 75 days of incubation. Except when the sucrose enrichment was 10 g/l and 20 g/l, where the root number and length after 75 days were substantially higher than that obtained after 60 days, the differences in root number and length between the 60 and the 75 days incubation regime at the other sucrose content was practically negligible. At all sucrose levels except 20 g/l, incubation for 75 days resulted in substantially higher rooting percentage than incubation for 60 days. If the plantlets height used as the sole parameter for selection, incubation period of 30 days and sucrose at 15 g/l would be recommended as the shotest possible incubation period for rooting of Moris. However, if all rooting aspect were taken in consideration, the best compromise between shortest incubation, lowest sucrose content and best rooting of Moris would be sucrose at 10 g/l and incubation for 75 days and sucrose at 15 and 20 g/l and incubation for 60 days. The 30 days incubation and sucrose at 30 g/l) could not be a choice at any case. After each incubation period, all of the plantlets, irrespective of its heights, rooting %, root number

and length and the sucrose treatments, were transferred to one tray of 75 cells filled with sand and garden soil mix and acclimatized under polyethylene enclosure. The plantlets survival was 83 % for the 30 and 45 days of incubation and 94 % for the 60 days incubation while all of the plantlets which rooted for 75 days survived acclimatization stage.

Sucrose was considered by several as cost factor of micropropagation. In fact, table sugar was suggested as cheaper sucrose alternative to lower the micropropagation cost of sugar cane (Yadav *et al*., 2004) and chrysanthemum (Belarmino and Gabon, 1999). On the contrary, calculation of the rooting cost per shoot (Table, 3.45; Figure, 3.24) showed that the different in rooting cost per shoot between the lowest and highest sucrose concentration was less than 1 cent and sucrose cost as percent of cost per shoot was only 1 %. Be and Debergh (2006) demonstrated that in tropical region, the electricity cost of incubation during multiplication and rooting stage of pineapple could be completely eliminated by outdoor incubation under lath house. Kodyan *et al*. (2001) reported that for banana cultures, diverting of natural light into enclosed room by using of tubulars could substitute for artificial light. These two approaches deserve further investigation and optimization. Cost and management of operations are the main obstacles of commercial application of micropropagation. However, the attention was always focused on reduction the cost by maximizing the rate and automation of multiplication stage (Firoozabady and Gutterson, 2003; Escalona *et al*., 1999). The rooting stage was almost ignored by all. In fact, in many cases the rooting was reported as general statement. If no attention paid to the rooting and remained conducted the way it is usually reported, complains about the high cost of micropropagated materials would always be there even if the multiplication was fully automated and at highest rate.

Table (3.43). P values of the main and interaction effect of sucrose concentrations and incubation periods on the in vitro rooting response of Moris pineapple cultures on liquid half strength medium enriched with IBA at 2.0 mg/l.

Factors	**df**	**Rooting parameters**			
		Plantlet height	Rooting %	Roots No.	Root length
Incubation periods	3	6.4E-08**	9.8E-06**	0.0500*	0.0002**
Sucrose	5	0.0015**	2.3E-10**	7.3E-07**	7.5E-05**
Incubation*Sucrose	15	0.8063	0.0879	0.4769	0.0054**
Error	48				
Total	72				

Table (3. 44). Effect of sucrose concentrations and incubation periods on the in vitro rooting of Moris pineapple cultured on liquid half strength MS medium enriched with IBA at 2.0 mg/l

Incub	**Sucrose concentrations** (mg/l)					
(Days)	5	10	15	20	25	30
Plantlet height (mm)						
30	15.7 h	23.0 defgh	21.7 defgh	22.3 defgh	20.3 efgh	22.0 defgh
45	19.0 gh	28.0 cdefgh	32.3 abcdefg	31.3 abcdefg	19.7 fgh	25.7 defgh
60	21.3 defgh	34.0 abcdefg	36.0 abcde	36.7 abcd	29.7 bcdefgh	32.7 abcdefg
75	24.3 defgh	35.3 abcdef	43.0 abc	43.7 ab	46.3 a	42.3 abc
Aver	20.1 B	30.1 A	33.3 A	33.5 A	29.0 A	30.7 A
Rooting %						
30	0.01 f	0.01 f	50 bcde	41.7 cdef	66.7 abcd	33.3 cdef
45	0.01 f	16.7 ef	75 abc	50 bcde	66.7 abcd	50 bcde
60	0.01 f	25 def	75 abc	91.7 ab	75 abc	66.7 abcd
75	0.01 f	100 a	91.7 ab	100 a	91.7 ab	75 abc
Aver	0.01 C	35.4 B	72.9 A	70.8 A	75.0 A	56.3 A
Roots/ shoot						
30	0.01 d	0.01 d	3.7 abc	2 abcd	2.7 abcd	2.3 abcd
45	0.01 d	1 cd	4 ab	1.7 bcd	3.3 abc	2.3 abcd
60	0.01 d	1 cd	3.3 abc	4 ab	2.7 abcd	2.7 abcd
75	0.01 d	4 ab	4.3 ab	4.7 a	3 abc	2.7 abcd
Avera	0.01 D	1.5 C	3.8 A	3.1 AB	2.9 AB	2.5 BC
Root length (mm)						
30	0.01 d	0.01 d	5.3 cd	5.7 cd	6 cd	4.7 cd
45	0.01 d	4.3 cd	7 bcd	3.7 cd	5.3 cd	5.7 cd
60	0.01 d	1.7 cd	10.7 bc	9.3 bcd	11.7 bc	8 bcd
75	0.01 d	16 b	10.3 bcd	27.3 a	10.7 bc	3 cd
Aver	0.01 C	5.5 B	8.3 AB	11.5 A	8.4 AB	5.3 B

Data were means of 15 shoots cultured on liquid, half strength MS medium enriched with IBA at 2.0 mg/l. Means followed by same letters were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range test.

Table (3.45). Effect of sucrose concentration and incubation period on the rooting cost per shoot of Moris pineapple cultured at density of **5** shoots per culture tubes containing 6 ml of liquid half strength MS medium enriched with IBA at 2.0 mg/l.

Incubation periods	**Sucrose concentration (g/l)**						
(Days)	5	10	15	20	25	30	Average
	Cost per shoot (RM)						
30	0.128	0.129	0.130	0.130	0.131	0.131	0.130
45	0.148	0.149	0.150	0.150	0.151	0.151	0.150
60	0.168	0.169	0.170	0.170	0.171	0.171	0.170
75	0.188	0.189	0.190	0.190	0.191	0.191	0.190
Average	0.158	0.159	0.160	0.160	0.161	0.161	0.160

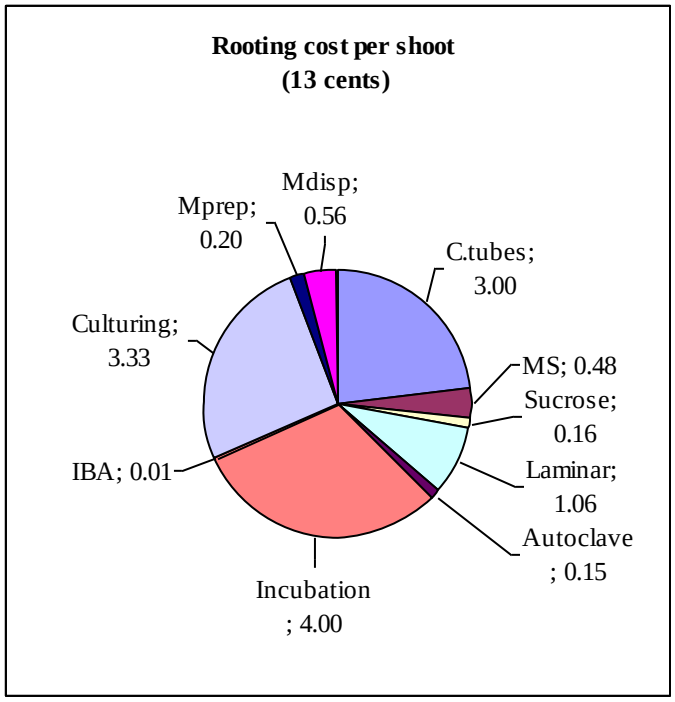


Figure (3.24). Rooting cost item per shoot of Moris pineapple. Shoots were cultured in **6** ml of liquid half strength MS medium enriched with sucrose at 15 g/l and IBA at 2.0 mg/l at density of five shoots per culture. Medium were autoclaved at 121 ^{0} C and 1.5 kg /cm^{2} for 20 minutes in glass jars and then dispensed into culture tubes. Cultures were incubated under constant temperature (25 ^{0} C and 16 hours of light provided by cool white florescence lamp for 75 days.
Mprep (Medium preparation); Mdisp (Medium dispensing); Ctubes (Culture tubes).
Culturing (separation and culturing of shoots under laminar).
Autoclave, Laminar, Incubation (electricity cost for operating autoclave, laminar and incubation room).

3.4. Acclimatization of Pineapple

3.4.1. Effect of peatmoss and sand mixing ratio on the acclimatization of Smooth cayenne pineapple

Table 3.46 showed that the plantlets survival as wells as the other growth parameters not only varied significantly at different mixing ratio of sand and peat moss but each parameter had its own optimal mixing ratio. The highest plantlets survival (100 %) obtained on sand alone and in mixing ratio of 1: 3 (25: 75) peat and sand. The tallest plantlets (120 mm), longest roots (46 mm) and heaviest plantlets (2.96 g) obtained in mixing ratio of 1: 4 (20: 80), 5: 3 (63: 33) and 1: 1 (50: 50 %) peat moss and sand respectively. The maximum folded leaves (11 leaves) obtained using peat moss alone (5: 0) while the maximum of 7 roots obtained using sand alone as well as in several combinations of peat and sands (5: 1, 5: 3 and 1: 1). However, the lowest plantlets survival (67 %) obtained at mixing ratio of 5: 3 (63: 37 %) and the shortest roots (31 mm) obtained at mixing ratio of 1: 3 (25 to 75 %) of peat moss and sand while the lowest response of all other parameters, plantlet height (103 mm), root number (5 roots), plantlets weight (2.42 g) was obtained in mixing ratio of 5: 4 (56 to 44 %) of peat moss and sand. Only two mixing ratio resulted in survival of all plantlets (100 %) while the highest percentage of dead plantlets (33 and 27 %) occurred in mixing ratio of (5 : 3) and (5 : 4) peatmoss and sand respectively. Peatmoss and sand at mixing ratio of (5 : 1), (5 : 2), (1: 1) and (1 : 2) resulted in 20 % death of plantlets and the plantlets that survived developed an intermediate level of growth.

The plantlets survival decreased from 93 to 73 % as the peat moss percentage decreased from 100 to 56 % and sand percentage increased from 0.0 to 44 %. Further decrease in peat moss up to complete elimination and increase in sand up to sand alone reversed the trend

of plantlets survival from decreasing to increasing to reach 100 % survival in mix consisted of 25 % peat moss and 75 % sand and pot filled of sand alone (Table, 3.46). The root length on the contrary, increased from 42 to 46 mm as the peat moss decreased from 100 to 63 % and sand increased from 0.0 to 37 % and decreased afterwards. The plantlet height and weight, on the other hand, was neither decreasing nor increasing as the peat moss decreased from 100 to 63 % and from 50 to 25 % and the sand increased from 0.0 to 37 % and from 50 to 75 %. However, both the plantlet height and weight were subjected to one sudden decline (116 to 103 mm and 2.83 to 2.42 g) occurred on the same mixing ratio when the peat moss decreased to 56 % and sand increased to 44 % and one sudden increase but at different mixing ratios. The plantlet reached its tallest height (120 mm) when the peat moss decreased to 20 % and sand increased to 80 % while the heaviest weight (2.96 g) obtained when the peat moss decreased and sand increased to 50 %. The highest root number (7 roots), on the other hand, obtained in peat moss and sand mixing ratio of 5: 1 (83: 17), 5: 3 (67: 37) and 1: 1 (50: 50) and when no peat moss was included in the mix (sand only). Of all substrates, sand alone was the most appropriate substrate for acclimatization of Smooth cayenne pineapple. The total cost for acclimatization of 30 plants in 6 pots full of sand was RM 18.26 which means that acclimatization of one plant cost 61 cents. However, because sand and pots are reusable items, the estimation of cost of acclimatization per plant was limited to cost of labor and the polyethylene sheet used for covering the pots and was 21 cents.

Survival is the most essential parameter of acclimatization and best index for selection of proper substrates type and mixing ratio. The low growth rate on the other hand could be handled by proper fertilization and irrigation schedule after transferring the survived plants to nursery bed or biger pots. Different mixing ratio of peat moss and sand resulted in significantly different plantlet survival and growth (Table, 3.46). The sand alone and mix

of sand at 75 % and peat moss at 25 % resulted in survival of all plantlets (100 %). However, contrary to our findings, Aydieh *et al*. (2000) reported that only 50 % of Queen pineapple plantlets survived in sand alone while 100 % on mix of 25 % sand and 75 % peat moss. Rahman *et al*. (2001) also reported that in coco peat alone all Kew pineapple plantlets survived while in mix of sand, cow dung and charcool only 70 % survived. Paz *et al.* (2000) obtained 100 % survival of Smooth cayenne using sand alone as substrate but under rigid control of environment. The substrate mixing percentage for optimal acclimatization might vary among cultivar and within plantlets of different quality and should be adjusted accordingly. Further investigation to identify these plantlets quality and proper substrate mixing percentage is needed. In this study, the plantlets used for acclimatization were 40 to 75 mm tall and the root number and length per plantlet ranged from 5 to 7 roots and 12 to 31 mm long and were rooted in agar solidified full strength MS medium enriched with NAA at 1.0 mg/l for 75 days. The rooting treatment (media and hormone and length of incubation period) affect the plantlets quality and consequently it expected to affect the proper mixing ratio of peat moss and sand.

The sand alone and mix of peat moss at 25 % and sand at 75 % (1: 3) resulted in survival of all plantlets (100 %) while in mix of 56 % of peatmoss and 44 % of sand (5: 4) and 63 % of peat moss and 37 % of sand ((5: 3) only 73 % and 67 % of the plantlets survived acclimatization respectively. The 33 % difference in survival clearly indicated that for commercial applicability of micropropation, selection of proper mixing ratio of substrate is very crucial. Comparing the survival and growth of plantlets at each two mixing ratios showed that small decrease or increase in the percentage of peat moss and sand in the mix could cause about 20 % loss of plantlets. On the other hand, it is interesting to note that equal percent of reduction of the mix components resulted in different acclimatization responses. Reduction of peat moss by 17 % (100 to 83 %) and increasing

of sand by 17 % (0.0 to 17 %) as in mixing ratio (5: 0) and (5 : 1) decreased the plants survival by 13 %, increased root length by 10 mm and had no effect on the plant weight while the same percentage (17 %) of reduction of peat moss (50 to 33 %) and increase of sand (50 to 67 %) using other mixing ratio of peat moss and sand (1: 1) and (1 : 2) had no effect on survival and root length but caused 7 % (0.42 grams) drop in the plant weight. Similar, reduction of peat by 8 % (71 to 63 %) and increasing of sand 8 % (29 to 37 %) as in mixing ratio (5: 2) and (5: 3) decreased the survival by 13 % and increased the root length by 6 mm while the same changes in percentage of mixing (33 to 25 peat moss and 67 to 75 sand) as in mixing ratio of (1: 2) and (1 : 3) increased the survival by 20 %, decreased root length by 9 mm and both had no effect on the plant weight. The optimal mixing percentage of peat moss and sand was critical and very narrow and each growth response had different optimal mixing percentage. Since the ratio of one substrate could not be possibly kept at one fixed percentage while using different percentage of the other substrate type, neither the main nor the interaction effect of the two substrates (peat moss and sand) on the survival and growth could be statistically analyzed. Nevertheless, analysis of the data using volume ratio as independent factor (Table, 3.47), ignoring the fact that the percentages of substrate at fixed volume of one substrate and different volumes of other were not equal, indicated that the plant weight was not affected by either substrates while the leaf formation was affected by sand and the plantlet height by peat moss. The plantlet survival, on the other hand, was affected mainly by the peatmoss portion of the mix and by interaction of the peat moss and sand while the root number and root length was affected only by the interaction of peat moss and sand. Estimation of the correlation indicated that the peat moss volume correlated negatively with survival and plantlet height and the sand correlated negatively with leaves formation.

Table (3.46). Effect of peat moss and sand mixing ratio on the acclimatization of Smooth cayenne pineapple

Rooting substrates		**Plantlets survival and growth**					
(Peat moss : Sand)		Survival	Plant height	Leaves	Root	Root	Weight
(Ratio)	(%)	(%)	(mm)	(No.)	(No.)	(mm)	(g)
5:0	100:0	93 ab	117 ab	11a	6 ab	42 ab	2.76 ab
5:1	83:17	80 bcd	109 ab	8 b	7 a	32 b	2.63 ab
5:2	71:29	80 bcd	110 ab	9 ab	6 ab	42 ab	2.74 ab
5:3	63:37	67 d	116 ab	10 ab	7 a	46 a	2.83 ab
5:4	56:44	73 cd	103 b	9 ab	5 b	39 ab	2.42 b
1:1	50:50	80 bcd	118 ab	10 ab	7 a	42 ab	2.96 a
1:2	33:67	80 bcd	117 ab	9 ab	6 ab	40 ab	2.75 ab
1:3	25:75	100 a	117 ab	10 ab	5 b	31 b	2.79 ab
1:4	20:80	87 abc	120 a	9 ab	6 ab	38 ab	2.68 ab
0:1	0:100	100 a	118 ab	9 ab	7 a	32 b	2.87 ab

Data represent mean of 30 plants that were rooted *in vitro* for 75 days in full strength agar solidified MS supplemented with sucrose at 30 g/l and enriched with NAA at 1.0 mg/l.
Means of the same column followed by same letters were not significantly different as tested by Duncan Multiple Range Test at $p \leq 0.05$.

Table (3.47). Significant of main and interaction effect of the rooting substrates on the acclimatization of micropropagated Smooth cayenne pineapples

Substrates	**df**	**Plantlets survival and growth**					
		Survival	Plant height	Leaves	Roots	Root	Weight
		%	(mm)	No.	No.	(mm).	(g)
				P values			
Peat moss	1	0.0224 *	0.0419 *	0.4208	0.8051	0.4357	0.1999
Sand	4	0.1543	0.8039	0.0493 *	0.1153	0.5572	0.3164
Peat*Sand	4	0.0432 *	0.4036	0.5738	0.0074 **	0.0131 *	0.5088
Error	20						
Total	30						

Plate (3.6) Smooth cayenne acclimatization on different mixing ratio of peatmoss and sand

Plate (3.7) Acclimatized Smooth cayenne pineapple

Different substrates have different physical and chemical properties and the plantlet survival and growth is a reflection of how the mixing ratio affected the physical and chemical property of the mix. The small changes of 8 % in mixing ratio are not expected to have profound effect on the percent of the easily available water (EAW) content of the substrate mix. However, it might cause a different in distribution of pore size within the substrate profile (container), the percent of air filled porosity (AFP), gaseous content and diffusion (Bilderback *et al*., 1982; Bunt, 1981; Paul and Lee, 1976) and the pH and electric conductivity of the mix (Aydieh *et al*., 2000). In this study, the pineapple that grown on peat moss and sand alone had better growth than those grown in mix of 5 parts of peat moss and 3 and 4 parts of sand. If the water content was a limiting factor, the plantlets that grown in sand only would had the lowest growth of all mixing ratios. The results (Table, 3.48) showed good plantlets growth on sand alone. Aeration seemed to be the most likely criteria that determined the proper substrate for pineapple acclimatization. On the other hand, Karlovich and Fonteno (1986) reported that duration of water stress rather than the EAW (easily available water) and AFP (air filled porosity) was the major cause of different growth response of Chrysanthemum. When the Chrysanthemum were irrigated before the water volume of the pot dropped below 500 ml, no difference in growth was observed irrespective of the media types and percent of EAW and AFR.

3.4.2. Effect of rooting treatments, plantlets quality and polyethylene cover on the acclimatization of Moris pineapple

Enclosing of the plantlets within polyethylene sheet increased the plantlets survival significantly (t 0.015) resulting in survival of 83 % of plantlets compared to 59 % of the uncovered plantlets while the medium states (t 0.33), IBA addition (t 0.24) and formation of roots (t 0.45) had no significant effect on the plantlets survival of acclimatization (Table, 3.48). IBA addition (Table, 3.49) had neither direct ($p \leq 0.40$) effect on the plantlets

survival nor interacted significantly with the medium states ($p \leq 0.205$). Medium states, on other hand, had no direct effect ($p \leq 0.229$) on the plantlets survival and did not interact significantly with the plantlets height ($p \leq 0.247$) and IBA ($p \leq 0.205$). However, it interacted significantly ($p \leq 0.061$) with the root number per plantlets (Table, 3.49). The percentage of plantlets survival after 60 days of acclimatization was mainly affected by the plantlet height ($p \leq 0.0003$) and to some extent by interaction between roots number per plantlet at the commence of acclimatization and the state of the medium ($p \leq 0.0618$) the plantlets were *in vitro* rooted in

Over 90 % of the plantlets that were taller than 41 mm survived the acclimatization irrespective of the state of the medium that were used for rooting (Table, 3.50). However, the survival of the plantlets shorter than 41 mm varied according to the plantlet height and the rooting medium state. At equal height of 30 and 40 mm, those rooted in liquid had higher survival percentage (57 and 78 %) than those rooted in solid (33 and 57 %). There were no significant different (t 0.4533) between the survival percentage of rootless and rooted plantlets (Table, 3.48) and survival of rooted plantlets ($p \leq 0.8177$) with unequal root number per plantlet at 0.05 level of significant (Table, 3.49). However, the survival was affected by interaction between the state of the rooting medium and number of roots per plantlets at 0.10 level of significant ($p \leq 0.0618$). Presence of more than 4 roots improved the survival percentage if the plantlets were rooted in solid and decreased the survival if the plantlets were rooted in liquid medium (Table, 3.50C). All plantlets (100 %) that rooted in solid medium and produced 6 roots survived acclimatization while the survival of the plantlets with 4 to 2 roots and those that failed to form roots (rootless) were 78, 67 and 56 % respectively. For the plantlets that were rooted in liquid medium, there was no significant different in the survival percentage (about 78 %) between rootless and rooted plantlets up to 4 roots per plantlet. However, about 50 % of the plantlets with 6

roots per plantlets failed to pass the acclimatization stage. The survival of rootless plantlets obtained from liquid rooting medium was 11 % higher than the rootless plantlets from solid rooting medium. On the contrary, the survival of plantlets with 6 roots per plantlets rooted in solid was 45 % higher than those rooted in liquid medium. The survival of plantlets with 2 to 4 roots per plants, on the other hand, were not significantly different whether it were rooted in solid or liquid medium. The effect of incubation periods of rooting stage was not tested in solid medium. However, the differences in the survival percentage after different rooting periods in liquid medium were significant ($p \leq 0.00001$). The results (Table, 3.50D) indicated that the longer the plantlets remained in the rooting medium the higher the percentage of survival during acclimatization. After 30 days of rooting, 71 % of the plantlets survived while after 75 days rooting almost all (94 %) of the plantlets survived the acclimatization. The survival percentage of the plantlets that rooted for 45 and 60 days was not significantly different and were intermediate (81 to 85 %) between that obtained after short rooting period of 30 and long period of 75 days.

The assessment of the optimal rooting treatment is usually judged by the percentage of rooting, number of roots per shoot and average root length without investigation the relation between rooting parameters, rooting treatments and the subsequent survival during acclimatization stage. The different IBA concentrations did have different effect on rooting parameters and about 44 % of shoots on the hormone free media failed to form roots, but the differences in plantlets survival percentage was not significant (Table, 3.48;Table, 3.50). Khan *et al*. (2004), Akbar *et al*. (2003) and Khatun *et al*. (1997) reported that none of the shoots rooted in hormone free and Bhatia and Ashwath (2002) reported that 70 % of the shoots did not root in hormone free medium. Yet, mentioned that over 88 % of the plantlets survived acclimatization. The t test (Table, 3.48) and analysis of variance (Table, 3.49) indicated that formation of roots, inclusion of IBA and states of medium were not

crucial for plantlets survival. The different in survival percentage of plantlets from hormone free and hormone contained, rootless and rooted plantlets and plantlets from solid and liquid medium were not significant. The height was the only plantlet quality that had great impact on the plantlets survival.

According to this study, survival during acclimatization depended mainly on covering the plantlets during acclimatization (Table, 3.48), the plantlet height at the time of acclimatization (Table, 3.49; Table, 3.50) and length of rooting period (Table, 3.50). Uncovered plantlets could survive under misting system (Paz *et al*., 2000) and inside growth chamber (Bhatia and Ashwath, 2002; Dewald *et al*., 1988), however, polyethylene cover and natural shade house conditions is much cheaper than installing, operating and maintenance of misting system and growth chamber. The plantlets used to test the covering effect were rooted in solid medium for 75 days and were not assorted according to their quality before covering. The 59 % survival of the uncovered plantlets of different quality indicated a possibility that plantlet of certain quality, height for instance, could survive in open pots under shade without cover requirement or being placed under mist system. Further work is needed to identify the best rooting quality for successful direct transfer of pineapple to open shade house.

The proper plantlet height for acclimatization, on the other hand, varied according to medium states used during *in vitro* rooting stage. If the plantlets were longer than 41, all survived acclimatization irrespective of the medium states during the *in vitro* rooting. If the plantlets were shorter than 41 mm tall, those which rooted in liquid medium had higher survival percentage than those rooted in solid medium (Table, 3.50). The states of the medium affected the rooting responses particularly the root length and plantlets height, but the difference in the survival of plantlets between the two states could not be detected (Table, 3.48) unless the plantlets were assorted into different height and root number

(Table, 3.50). The effect of root number per plantlet on the survival of the plantlets during acclimatization depended on the medium states. Shoots rooted in solid medium had different optimal root number for acclimatization from those rooted in liquid medium. The survival of the plantlets rooted in solid increased and those rooted in liquid decreased as the roots per plantlet increased to 6 roots per plantlet (Table, 3.50). Plantlets (rooted shoots) of different height, 30 (Fitchet, 1990a), 40 (Sripaoraya *et al*., 2006), 50 (Ko *et al*., 2006), 70 (Vesco *et al*., 2001), 80 (Kiss *et al*., 1995) that were rooted in solid and 90 mm (Be and Debergh, 2006) that were rooted in liquid medium survived acclimatization. Rootless shoots longer than 30 (Dewald *et al*., 1988) and 50 mm (Fernando, 1986) could directly transferred from liquid multiplication medium and rootless shoots longer than 80 mm (Escalona *et al*., 1999) could be transferred from liquid elongation medium to *ex vitro* acclimatization. The plantlets survival increased from 71 to 94 % as the incubation period in the rooting media increased from 30 to 75 days (Table, 3.50D). However, longer incubation would increase the electricity bill and medium solidification would increase the medium cost. Addition of hormones and agar solidification of medium during *in vitro* rooting stage was not essential for survival of Moriss pineapple plantlets during acclimatization. Any combination of solid medium and short incubation and liquid medium and long incubation that could result in plantlets longer than 41 mm but with 6 roots in solid and less than 4 roots in liquid would combine lower cost of rooting stage and highest survival during acclimatization but at different time of propagules delivery. In conclusion, it is clear that the plantlets height is an important quality for acclimatization. However, plantlets height is not direct factor, it only a monitor of when the acclimatization should be done. The real factor is an effective control that the plantlets probably had over water loss once it reached that height. The importance of plantlets height and longer incubation and their relation with medium states for plantlet survival of acclimatization

Table (3.48). Significant of effect of medium states and IBA concentrations during *in vitro* rooting and root per plantlet and polyethylene cover during acclimatization on the Moris pineapple plantlets survival of acclimatization

Factors		Sample	Survival	t	Confidence interval (95 %)	
		Size (n)	%	Test	lower	Upper
Polyethylene sheet	Covered	75	83			
	Uncovered	75	59	0.0146	0.2552	2.145
Medium states	Liquid	50	80			
	Solid	50	72	0.3306	-0.7706	2.1706
IBA	Contained	90	76			
	Free	18	61	0.2406	-1.022	3.622
Roots	Rooted	54	78			
	Rootless	18	67	0.4533	-2.0518	4.0518

Table (3.49). Significant of IBA concentrations and medium states during *in vitro* rooting and plantlets height and root number at the commence of acclimatization on the Moris pineapple survival of acclimatization.

Factors	df	P	Factors	df	P
IBA concentrations and medium states			**Plantlet height and medium states**		
IBA concentrations	5	0.8278	Plantlet height	3	0.0003**
Medium states	1	0.4000	Medium states	1	0.2295
States*Conc.	5	0.2054	States*Height	3	0.2476
Error	24		Error	16	
Total	36		Total	24	
Root per plantlet and medium states					
Roots number	3	0.8176			
Medium states	1	0.7565			
States*Roots	3	0.0618			
Error	16				
Total	24				

Table (3.50). Effect of IBA concentration, medium states, incubation periods and rooting parameters (plantlets quality) on the survival of Moris pineapple during acclimatization

Factors	**Medium states**			**Factors**	**Medium states**		
	Solid	Liquid	Average		Solid	Liquid	Average
A. IBA conc. (mg/l)				**B. Plantlet heights** (mm)			
	Survival %				Survival %		
0	77.7	44.3	61	25 - 30	33 c	55.67 bc	44.33 c
0.5	78	66.7	72.3	31 - 40	55.67 bc	78 ab	66.83 b
1	44.3	78	61.2	41 - 50	89 a	89 a	89 a
1.5	78	89	83.5	51 - 60	100 a	89 a	94.5 a
2	89	77.7	83.3				
2.5	67	89	78				
C. Roots per plantlet.				**D. Incubation periods** (days)			
	Survival %				Survival %		
0	67 ab	67 ab	67	30	Nt	71 b	
2	55.67 b	89 ab	72.3	45	Nt	80.5 ab	
4	78 ab	77.67 ab	77.8	60	Nt	84.5 ab	
6	100 a	55.33 b	77.7	75	Nt	94.25 a	

Means followed by same letters were not significantly different at probability of 0.05 according to Duncan Multiple Range test

Table (3.51). Estimated total cost of pineapple acclimatization and the effect of polyethylene enclosure on plantlets survival

	Plantlets		Cost items (RM)					Cost /plant	
	Used	Survived	Sand	Sheets	Labor	Pots	Total	Include	Minus
Smooth cayenne									
Covered	30	30	4.51	0.25	6.00	7.5	18.26	0.609	0.21
Moris									
Coverd	75	75	12.82	0.5	3.33	14.75	31.40	0.419	0.05
Uncovered	75	44	12.82	0.0	3.33	14.75	31.40	0.714	0.09
Covered	90	90	15.38	0.5	4.00	17.70	37.58	0.418	0.05

Include (Cost estimation include pots and sand cost)
Minus (Cost estimate did not include pots and sand cost)
RM (1 USA= RM 3.25)

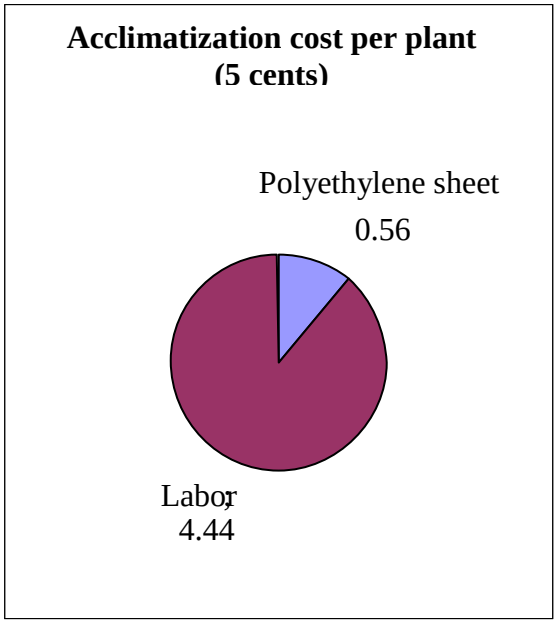


Figure (3.25). Acclimatization cost per plantlet of pineapple using large pots (10 inch) filled with sand and 15 plantlets per pot. Each 6 pots were placed under polyethylene sheet enclosure. (cost of pots, and sand were not included because they are reusable items)

Plate (3.8). Acclimatization of Moris pineapple

Plate (3.9) acclimatized Moris pineapple

indicated that the plantlets had undergone some sort of *in vitro* hardening. Hence, functioning stomata and wax deposit could be better parameters for evaluation of rooting treatments than the commonly used rooting percentage, number and length. The cost of acclimatization not including the sand and pots cost (reusable items) was 5 cents per plant (Table, 3.51; figure, 3.25).

Summary and Conclusion

Series of 25 experiments were conducted to test the effect of several factors such as hormone types and concentrations, incubation periods, subcultures, medium states, sucrose, pH, explants size, explants density and medium volume per culture, and medium strength on the shoot formation rate per explant, total shoots, total cost and cost per shoot at each and over 4 consecutive subcultures and the total shoots per liter of medium. Rooting responses and plantlets quality of Smooth cayenne and Moris pineapple were tested in different hormones, media strength, shoots age, sucrose content and incubation periods. Survival during acclimatization was evaluated in relation with rooting treatments, plantlet quality and substrate types and mixing ratio under polyethylene enclosure. The cost at each stage of multiplication, rooting and acclimatization was partition into the various cost items to determine the major cause of cost at each stage. The objectives were to optimize the shoot formation rate and total shoot production, minimize the total cost and cost per shoot during multiplication and rooting stages and to identify the major cost factor and provide means of better management of mass propagules production that could fit small company with limited budget, facility and market access and big company with unlimited resources and world wide connections.

The effect of 7 different hormone treatments on shoot formation and growth pattern of Smooth cayenne at each two weeks over 105 days incubation and the shoot formation per explant and total shoots at 4 incubation periods (30, 45, 60 and 75 days) and over 4 subcultures were investigated. The shoot formation occurred in alternative flushes of low and high shoot formation at each 15 to 30 days, the magnitude and time of each one varied depending on the hormone treatments. Hence, at different incubation periods there were different optimal hormone treatment. The hormone treatment in which the highest cycle of

shoot formation occurred before termination of incubation was the optimal for that incubation period and those in which termination occurred before the pulse take place resulted in less shoot formation. In term of shoot formation per explant, the best hormone treatment for Smooth cayenne varied at different incubation period. After 30 days of incubation the best treatments were combination of BAP at 2.25 mg/l and IAA at 0.75 mg/l and BAP at 3.25 and IAA at 1.75 mg/l. Over 4 subcultures both treatments resulted in formation of 6 shoots per explant and the difference in total shoots were not significant but at different cost per shoot (24 and 27 cents, respectively). After 45 days, the best hormone treatments were BAP singly applied at 2.25 mg/l and combination of BAP at 3.25 and IAA at 1.75 mg/l. Both resulted in formation of 10 shoots per explant and the different in total shoots were not significant but the different in cost per shoot were too high reaching 10 cents per shoot (15 and 25 cents, respectively). In both regimes of incubation periods the cost per shoot at the best hormone treatments was unaffordable and could not be adopted for commercial production of propagules. After 60 days, four hormone treatments resulted in equal shoot formation rate (14 shoots) but at different total shoots and cost per shoot. The highest total shoots (32,256 shoots) and lowest cost (11 cents) obtained using BAP at 2.25 and IAA at 0.75 mg/l. The difference in total shoots between treatments of equal shoot formation rate per explant was 5,000 to 11,000 shoots and the different in cost per shoot was 3 to 5 cents per shoot. Similar, after 75 days, four treatments resulted in equal rate (18 shoots per explant) but the highest total shoots (121,125 shoots) and the lowest cost per shoot (8 cents) obtained using BAP at 3.25 and IAA at 1.75 mg/l. The difference in total shoots between treatments of equal rate was 29000 to 53000 shoots and the different in cost per shoot was 2 to 4 cents per shoot. Using of total shoots for assessment is very suitable approach to select the most suitable treatment. Analysis of cost at each subculture showed that the cost per shoot varied at each subculture. Using combination of

BAP at 3.25 mg/l and IAA at 1.75 mg/l and incubation of 75 days, the total shoots after 4 subcultures were 121,125 shoots and the cost per shoots was 8 cents. However, the total shoots after three subcultures were 8075 shoots and the cost per shoot was 5 cents only. Hence, instead of starting with one explant and subculturing for four times, starting with 15 explants (shoots) and subculturing for three subcultures would produce the same total of shoots (121,125 shoots) but at lower cost per shoot (5 cents).

Single application of BAP was tested at 15 concentrations. Highest rate of 12 shoots was obtained at 4 different concentrations. The effect of different explants density and shoot clusters of different size on the shoot formation and total shoots per liter were compared. Using of higher explants density decreased the shoot formation capacity from 11 shoots at density of one explant to 7 shoots at density of four explants. However, the higher explants density doubled the total of shoots per liter and lowered the cost per shoot compared to density of one explant per culture.

Multiplication of Moris, on the other hand, was tested at density of one explant per culture in 20 ml of solid full strength MS medium enriched with sucrose at 30 g/l and medium pH adjusted to 5.7 using 4 different hormones (BAP, KN, NAA and IAA) applied singly at 10 different concentrations for three subcultures. The highest average of shoot formation over three subcultures (6 shoots per explant) obtained in cultures enriched with BAP, NAA and IAA singly applied at 2.0 mg/l and BAP and IAA singly applied at 3.0 and 3.5 mg/l respectively. The total shoots identified BAP at 3.0 mg/l as the best treatment with highest total shoots (210 shoots in three subcultures). However, the cost per shoot was too high (25 cents). At each of the three subcultures there were different optimal hormone type and concentration for highest shoot formation. The highest shoot formation at the first, second and third subculture (7, 12 and 7 shoots) obtained in media enriched with BAP at 3.0, IAA at 2.0 and 3.5 and NAA at 2.5 mg/l respectively. Keeping the sucrose at

30 g/l and the medium volume per culture at 20 ml in medium enriched with BAP at 2.0 mg/l but lowering the medium pH to 5.0, using of both solid and liquid media resulted in equal rate of 7 shoots, total of 350 shoots per liter at total cost of 71 to 76 RM and reduced the cost per shoot to 14 and 13 cents. The effect of the 4 hormones (BAP, KN, NAA and IAA) each at 10 different concentrations were retested in liquid medium at density of 2 explants, lower medium volume per culture (10 ml), sucrose at 20 and pH 5.0. BAP at 2.0 mg/ l was the best hormone resulting in formation of 8 shoots per explant, total shoot of 1,600 per liter and total cost of RM 91.3 and the cost per shoot reduced to 6 cents.

To decrease the cost per shoot below 6 cents and increase the total shoots per liter above 1,600 shoots, the BAP and sucrose of the medium were fixed at 2.0 mg/l, 20 g/l and the pH at 5.0 and explants of different size at different explants density and medium volumes per culture were tested over different incubation periods (30, 45, 60 and75 days) for 4 subcultures. During multiplication, shoots of different size ranged from 5 to 40 mm long and different frequency were produced. The shoot formation capacity of the shoots of different sizes (5, 10, 15, 20, 25 mm) were tested at different explants density (1, 2, 3, 4, 5 shoots) per culture using liquid medium enriched with BAP at 2.0 mg/l, sucrose at 20 g/l, adjusted to pH 5.0 and dispensed at 10 ml per culture. Shoots of different size had different shoot formation capacity and different optimal density per culture. Longer shoots had higher shoot formation capacity than shorter shoots and shoots of different size responded differently to different explants density per culture. The highest shoot formation of the 20 and 25 mm long shoots (7 and 8 shoots) obtained at density of one shoot per culture while for the 5, 10 and 15 mm long shoots the highest rate (4, 6 and 7 shoots) obtained using 5, 2 and 4 shoots per culture. However, the highest total per liter from the 5 and 25 mm long shoots (2,000 and 2,667 shoots) obtained using density of 5 shoots per culture and for 10, 15 and 20 mm long shoots density of 4 shoots per culture resulted in the highest total per

liter (1,866, 2,800, 2,667 shoots). The cost per shoot in all cases was 3 to 4 cents per shoot. At density of 4 explants and 10 ml per culture, using of 15 mm long shoots produced the highest total shoots per liter (2,800 shoots) at total cost of RM 90.85 and 3.24 cents per shoot. Using of only one and two shoots per culture resulted in highest rate per explants but less total per liter and higher cost per shoot (12 and 8 cents respectively). It clear that using the same treatment for shoots of different size and assessment of different treatments based on shoot formation per explant simplify the procedure but not suitable for optimal production of shoots at lowest cost per shoot.

Different explant densities were tested at different medium volumes per culture and different incubation periods over 2 to 4 subcultures and different medium volumes per culture were tested at different media strength and pH adjustments. Out of 48 combinations of 4 incubation periods (30, 45, 60 and 75 days), 3 explants density (1, 2 and 3 shoots per culture) and 4 volumes of medium per culture (3, 6, 9 and 12 ml), 5 combinations (density of 1 explant in 3 and 9 ml of medium per culture for 75 days, density of 3 explants in 9 and 12 ml of medium per culture for 75 days and density of 3 explants in12 ml of medium for 60 days incubation) resulted in the highest shoot formation per explant (11 shoots) but the cost per shoot of the first two combinations was too high (9 cents). The cost per shoot of the other three combinations was low (3 cents) but the total shoots per liter were very low (3,667; 2,750 and 2,750 shoots).

Using of 3 explants and 3 ml of medium per culture and incubation for 75 days resulted in the highest total of shoots (6,000 shoots) but at high total cost (RM 305) and high cost per shoot (5 cents). Hence using of 3 explants and 6 ml of medium per culture and incubation for 75 days (5,000 shoots and RM 159 per liter and 3 cent per shoot) was the best compromise between highest total shoots and lowest total cost and lowest cost per shoot. At all incubation periods the explants density had to be more than 2 explants per

culture for the cost per shoot to be less than 8 cents. Using of density of 1 and 2 explants, the cost per shoot ranged from lowest of 8 to highest of 16 cents depending on the medium volume and the incubation periods. However, using of 3 explants per culture reduced the cost per shoot to affordable level but the medium volume per culture had to be more than 6 ml per culture. The lowest cost after 30, 45, 60 and 75 days of incubation was 5, 4, 3 and 3 cents obtained when the medium dispensed at 9, 9, 12 and 6 ml per culture respectively. Dispensing the medium at 6 ml per culture and using of 3 explants per culture the cost per shoot after 30, 45, 60 and 75 days was 7, 6, 5 and 3 cents respectively. Incubation for 30 and 45 days irrespective of explants density and medium volume per culture produced lower rate per explant, less total shoots per liter and resulted in higher cost per shoot than incubation for 60 and 75 days.

Testing the explants density using 10 instead of 4 different volumes per culture and fixed incubation of 75 days showed that medium volume as low as 4 ml per culture could be used for effective propagation of pineapple. At fixed incubation of 75 days, using of one explant in 3, 7 and 9 ml of medium per culture and 3 explants in 9 and 12 ml of medium per culture resulted in highest rate of shoot formation per explant (11 shoots) but the cost per shoot at density of one explant was 9 cents and at density of three explants was 3 cents and all resulted in lower total shoots per liter (3,667; 1,571; 1,222; 3667 and 2750 shoots). Using of 3 explants in 3 ml of medium per culture, on the other hand, resulted in low rate of shoot formation (6 shoots per explant) but the second highest total of shoots per liter (6000 shoots) and the cost per shoots was 5 cents. The highest total per liter (7500 shoots) and lowest cost per shoot (3 cents) obtained using 3 explants and 4 ml of medium per culture but at highest total cost per liter (RM 232). The lowest cost per shoot (3 cents) obtained using 3 explants and four different volumes of medium per culture (4, 6, 9 and 12 ml) but at different total of shoots (7,500; 5,000; 3,667 and 2750 shoots) and different total

cost (RM 232, RM 159, RM 111 and RM 87). Selection of either one of these treatments is a management decision based on budget and target total shoots and time of production.

Using medium strength 2.0x dispensed at 7 ml per culture, resulted in the shoot formation per explant but lower total shoots (4,286 shoots) and total cost of RM 129 while dispensing the medium at 4 ml per culture resulted in one shoot less per explant (9 shoots) but highest total shoots per liter (6,750 shoots) at cost of RM 211 and both resulted in equal cost per shoot (3 cents). Increasing the medium strength from 0.5 x to 1.0, 1.5x and 2.0x increased the total shoots and total cost per liter and decreased the cost per shoot while increasing the medium volume per culture decreased total shoot and total cost per liter and increased the cost per shoot. Dispensing the medium at 4 ml per culture, increasing the medium strength from 0.5 x to 1.0, 1.5x and 2.0x increased the shoot formation per explant from 3 to 5, 7 and 9 shoots, the total shoots per liter from 2,250 to 3,750; 5,250 and 6,750 shoots, total cost from RM 199 to RM 203, RM 207 and RM 211 per liter and decreased the cost per shoot from 9 to 5, 4 and 3 cents. Lowest cost per shoot (3 cents) obtained using full strength (1.0x) dispensed at 6 ml, one and half strength (1.5x) dispensed at 7 and 8 ml and double strength (2.0x) dispensed at 4, 6, 7 and 8 ml per culture but at different total shoots and total cost per liter. The highest total shoots (6750 shoots) and cost (RM 210) per liter of medium was obtained dispensing the 2.0x strength medium at 4 ml per culture while the lowest total shoots (3000 shoots) and cost (RM 112) obtained dispensing the 1.5x strength medium at 8 ml per culture. Dispensing the 1.0 x strength medium at 6 ml per culture resulted in intermediate total shoots (4000 shoots) and cost (RM 139 RM) and was best compromise between highest total shoots and lowest total cost per liter of medium.

Testing the multiplication of Moris at density of 1, 2, 3 and 4 shoots in 4, 6 and 8 ml of medium per culture for four subcultures showed that treatments with highest rate of shoot

formation per explant and total shoots over consecutive subcultures was not the best for production of shoots at lowest cost. The highest shoot formation per explant (6 shoots) and total shoot (504 shoots) after two subcultures obtained using 3 and 4 explants and 8 ml of medium per culture and the cost per shoot was 4 cents. The highest total shoots (3780 and 22680 shoots) after three and four subcultures obtained using one explant and 8 ml of medium per culture but the cost per shoot was 12 and 17 cents and the total cost was RM 459.6 and RM 3,819.5 respectively. Using of 4 explants and 8 ml of medium per culture, on the other hand, resulted in lower total shoots (3024 and 15120 shoots) but the cost per shoot was reduced to 4 and 5 cents and the total cost was RM 134.3 and RM 806.5 respectively. Analysis of cost items indicated that the major cause of cost was electricity during the incubation period (45 %) followed by labor wages (22 %) and culture tubes (17 %).

Two auxins (IBA and NAA) each singly applied at 5 different concentrations were tested for their effect on rooting of Smooth cayenne shoots of different age using 20 ml of agar solidified full strength MS enriched with sucrose at 30 g/l and 60 days of incubation. Both of hormones and shoots age had no significant effect on rooting percentage. However, for the other rooting responses NAA at 1.0 mg/l was the best hormone treatment. Older shoots treated with NAA at 1.0 mg/l produced longer roots and taller plantlets than younger shoots while younger shoots produced more roots than older shoots. However, the cost of rooting was too high (101 cent per shoot). Testing the same hormone treatments on rooting of different cultivars (Smooth cayenne and Moris) at different media strength (Quarter, Half and Full strength MS) and lower medium volume per culture (10 ml) indicated that different cultivars had different rooting hormone and the different rooting parameters had different optimal concentrations of hormone and media strength. The best hormone for Smooth cayenne was NAA at 1.0 mg/l but full strength resulted in tallest

plantlets and more roots per shoot while quarter strength resulted in longer roots and higher percentage of rooting. The best hormone for Moris, on the other hand, was IBA at 2.0 mg/l but the tallest plantlets and longer roots obtained in quarter strength while higher rooting percentage on half strength and more root formation per shoot obtained on full strength hormone free medium. Using of quarter strength medium and lower medium per culture (10 ml) reduced the cost of rooting to 91 cents per shoot but the cost still unaffordable. Analysis of cost items showed that the major cause of cost was electricity of incubation (44 %) followed by labor wages (23 %) and cost of culture tubes (17 %).

Using different shoot sizes (5, 10, 15 and 20 mm) at different shoots density per culture (1, 2, 3, 4 and 5 shoots) in 10 ml of agar solidified full strength MS medium enriched with sucrose at 30 g/l showed that the rooting capacity of the shoots was not affected by the shoots size and shoots density per culture. At all shoots size and density per culture the differences in rooting percentage, root formation per shoot and root length was not significant. However, longer shoots produced taller plantlets. The 5 and 10 mm long shoots resulted in shorter plantlets (39 and 50 mm tall) and 15 and 20 mm long shoots on taller plantlets (57 mm tall). Increasing the density of shoots, on the other hand, from 1 shoot to 5 shoots per culture reduced the cost of rooting per shoot from 101 to 19.5 cents. Testing of different sucrose concentration (10, 20, 30 and 40 g/l) and pH adjustments (5.0, 5.5, 6.0 and 6.5) in solid and liquid full strength MS medium at fixed explants density of 3 shoots and medium volume of 8 ml per culture indicated that the rooting capacity was affected by sucrose and medium states. The pH adjustments, on the other hand, had no significant direct effect in all rooting parameters but interacted significantly with sucrose and had no significant interaction with medium states. All shoots rooted in liquid medium enriched with sucrose at 20 g/l and adjusted to pH 5.0 and resulted in tallest plantlets (71 mm tall). But more (11 roots per shoot) and longer roots (39 mm) obtained in liquid medium

enriched with sucrose at 30 g/l. The rooting cost per shoot was 29.5 cents. Although had significant effect on rooting parameters, the different sucrose at fixed medium state and the different media state at fixed sucrose had little influence on rooting cost. The different in rooting cost per shoot between different combination of sucrose and media state was less than one cent. Analysis of the cost items indicated that the major cause of cost was electricity of incubation, labor wages and cost of culture tubes.

Testing of 6 different sucrose concentrations (5, 10, 15, 20, 25 and 30 g/l) and different incubation periods (30, 45, 60 and 75 days) using 6 ml of half strength MS medium and 5 explants per culture indicated that the best rooting obtained using intermediate sucrose concentrations (15- 20 g/l) and longer incubation period (75 days). At all incubation periods, no root formation occurred in medium enriched with sucrose at 5 g/l and the plantlets were the shortest (20 mm). At all sucrose concentrations, only 50 % of the shoots rooted during the first 30 and 45 days while 91 % rooted after 60 and 75 days. At any sucrose concentration using of longer incubation period resulted in taller plantlets and higher rooting percentage than shorter incubation. However, incubation longer than 60 days did not increase the root number per shoot and root length except at 20 g/l where incubation for 75 days resulted in the longest roots (39 mm). In medium enriched with 25 and 30 g/l, all incubation periods had no effect on root number per shoot. On the other hand, at shorter incubation of 30 and 45 days, using of sucrose higher than 15 g/l did not improve the plantlet height and root number and sucrose higher than 25 g/l did not improve the rooting percentage. At incubation of 60 days, sucrose higher than 20 g/ did not improve any of the rooting parameters. At incubation of 75 days, using of sucrose higher than 20 g/l did not improve root formation and root length but using of 25 g/l resulted in taller plantlets and higher rooting percentage than sucrose at 20 g/l. Although sucrose was important for rooting of Moris, the sucrose concentrations had very little effect on cost of

rooting. At fixed incubation period the difference in rooting cost per shoot of shoots rooted in different sucrose concentrations was less than one cent. The rooting responses as well as cost was affected by incubation periods more than by the sucrose concentrations. In other words using of sucrose concentration higher than 20 g/l is just wasting of money and longer incubation period increased the rooting percentage and plantlets higher but increased also the rooting cost per shoot by up to 6 cents than shorter incubation. The rooting cost after 30 days of incubation was 13 cents while incubation for 45, 60 and 75 increased the cost to 15, 17 and 19 cents respectively.

Two substrates (sand and peatmoss) at 10 mixing ratio were tested for acclimatization of Smooth cayenne. Using of sand alone and mix of sand and peat moss at ratio of 3: 1 resulted in 100 % survival of plantlets. Acclimatization of Moris, on the other hand, were tested in relation to IBA concentrations, medium states and incubation periods used during rooting stage, rooting parameters and plantlets quality at the time of acclimatization and to using of polyethylene cover. Covering of the plantlets with polyethylene sheet, lengths of incubation period during rooting and plantlets height were very essential for survival of plantlets during acclimatization. All of the plantlets that were covered with polyethylene sheet during hardening survived acclimatization while only 59 % of the uncovered plantlets survived acclimatization. All of plantlets that were *in vitro* rooted for 60 and 75 days survived acclimatization while only 84 % of those *in vitro* rooted for 30 and 45 days survived acclimatization. Irrespective of whether shorter or longer incubation period, liquid or solid media were used for *in vitro* rooting and whether rooted or rootless plantlets were used for acclimatization, over 91 % of plantlets that were taller than 41 mm survived acclimatization. On contrary, not only low percentage (55 %) of the plantlets that was shorter than 41 mm survived acclimatization but also their survival depended on the medium states they rooted in and the number of roots per plantlet. It is interesting that

formation of more roots increased the survival of shoots which were rooted in solid but decreased the survival of shoots that were rooted in liquid medium. The pineapple propagules are usually soled bared without soil ball around the roots and can be stored for 2 to 3 months. Therefore, the cost of sand and pots were included in estimation of total cost but was not included in estimation of cost per plantlets. The estimated acclimatization cost using 15 plantlets in each pot filled of sand and group of 6 pots under enclosure of polyethylene sheet was 5 cents per plant.

Out of these experiments of multiplication, rooting and acclimatization it could be concluded that:

- Different pineapple cultivars had different optimal treatments for multiplication and rooting and one treatment could not be generalized for the others.
- At different incubation periods and different subcultures, the same cultivar had different requirement of hormone concentration, explant density and medium volume per culture for optimal multiplication and rooting.
- *In vitro* shoot formation of pineapple occurred in alternative cycles of high and low shoot formation each 15 days. The timing of each cycle varied according to the hormone treatment.
- For higher shoot formation and low cost per shoot, the incubation period should be longer than 45 days.
- Shoot of different sizes had different shoot formation but equal rooting capacity
- The effect of explants density on the shoot formation per explant varied among explants of different size, medium volumes per culture, incubation periods and subcultures. Higher explant density suppressed the shoot formation of larger shoots ($\geq$ 20 mm) but promoted that of smaller shoots ($\leq$ 10 mm).

- Rate of shoot formation per explant was not an effective tool for assessment of commercial applicability of micropropagation protocol. Treatments with high rate resulted in low total shoots per unit of medium and high cost per shoot. Total per liter was more practical and effective for identification of treatments with high total shoots and low cost.
- Medium as low as 4-12 ml per culture could be used for multiplication and rooting of pineapple.
- Optimal shoot formation in solid medium required higher sucrose enrichment than liquid medium.
- The effect of medium strength depended on the medium pH and medium volume per culture. At pH 5.0 and 5.5, double strength medium (2.0x) resulted in higher while at pH 6.5 resulted in lower shoot formation per explant than medium that obtained in medium strength 1.0x and 1.5x.
- Shoot age, cultivars and sucrose treatments were more important for rooting than application of hormone. Shoots from 10 months old culture stocks produced more roots than that from 6 month old shoots and no rooting occurred in medium enriched with sucrose at 5 g/l.
- Different rooting parameters had different optimal hormone concentrations and media strength.
- In both stages of multiplication and rooting, sucrose and agar were not major cost factors of micropropagation. The major cost factors were electricity of incubation period followed by labor and culture tubes.
- Rooting stage was the most costly stage of micropropagation

- Survival of plantlets during acclimatization depended mainly on plantlets height, polyethylene cover and length of *in vitro* rooting period. The longer incubation and taller plantlets, the higher acclimatization survival percentage.

In this project almost all of the factors that affect the shoot formation, rooting and cost were investigated and two different protocols for commercial propagation were developed for Smooth cayenne and Moris pineapple. A total of 121,125 planting materials of Smooth cayenne could be produced per year from single shoots at cost of 26 cents per propagules (Table, 4.52). Single shoot underwent 4 multiplication cycles each 75 days long on 20 ml of agar solidified full strength MS enriched with sucrose at 30 g/l, combination of BAP at 3.25 and IAA at 1.75 mg/l and pH adjusted to 5.7. The total shoots, total cost and cost per shoot at the first and after 2, 3 and 4 subcultures were 17; 323; 8075 and 121125 shoots, RM 1.61; RM 28.63; RM 542.82 and RM 13,437.36 and 6.5, 6.2, 4.7 and 7.8 cents respectively. A total of 15,120 planting materials of Moris per year could be produced starting with 12 shoots at cost of 23 cents per propagule (Table, 4.53). Shoots underwent 4 multiplication cycles each 60 days long on 8 ml of liquid full strength MS enriched with sucrose at 20 g/l, BAP at 2.0 mg/l and pH adjusted to 5.0. The total shoots, total cost and cost per shoot at the first and after 2, 3 and 4 subcultures were 84; 504; 3024 and 15,120 shoots, RM 2.98; RM 21.96; RM 134.26 and RM 806.51 and 4; 4; 4 and 5 cents respectively.

The shoots of both cultivars (Smooth cayenne and Moris) were rooted at density of 5 shoots per culture in 6 ml of quarter strength liquid MS enriched with sucrose at 20 g/l, but enriched with different hormones. NAA at 1.0 was used for rooting of Smooth cayenne and IBA at 2.0 mg/l for rooting of Moris, pH adjusted to 5.0 and the cultures incubated for

30 days. The plantlets of both cultivars were acclimatized in pots filled with sand under polyethylene enclosure for 21 days. Then the cover removed and plantlets kept under shade for 75 days. The cost of rooting was 13 cents per shoot and that of acclimatization was 5 cents per plantlet. The total cost of rooting of Smooth cayenne was RM 15,254.36 and that of Moris was RM 1,904.85. Analysis of cost items showed that the major cause of cost on both multiplication and rooting was electricity of incubation, labor wages and cost of culture tubes (Table, 4.52 and 4.53) and rooting stage was the costly stage (Figure, 4.26).

Although production cost of about 23 cents per propagules is reasonable, further investigation particularly of the incubation period conditions and testing of new approaches to bypass rooting stage could reduce particularly the cost of rooting below 13 cents per shoot.

- The major cost factors of both multiplication and rooting stages was electricity of incubation followed by labor wages and culture tubes and the rooting stage was the costly stage.
- Cost of electricity of both stages could be reduced or even completely eliminated by adopting of outdoor incubation under shade house, incubation under room conditions, use of shorter incubation period, shorter photoperiods and higher temperature and reducing the shelving space by using of higher shoots density per culture.
- Rooting stage could be bypassed by developing treatment for simultaneous multiplication and elongation, simultaneous multiplication and rooting and by *in vitro* root induction using high pulse rooting treatment and *ex vitro* root development under hardening and acclimatization conditions.
- Testing the effect of explants density on the rooting ability of shoots of different size indicated that the density had no significant effect on the rooting ability of the shoots.

Using of higher density than the 5 shoots per culture used in this study would reduce the cost of culture tubes and reduce the shelving space and electricity cost

- Reusing of the culture tubes during multiplication by descanting and adding the medium under laminar and reusing of the culture tubes used during multiplication for rooting stage could reduce the cost of culture tubes.

Table (4.52). Total cost and cost items of micropropagation stages (multiplication, rooting and acclimatization) of Smooth cayenne pineapple starting by one shoot in Malaysian Ringgit (RM)

Cost items		Multiplication	Rooting	Total	%	Cost items	Acclimatization
Jars		3876.00	0.00	3876.00		Pots	23821.25
C.tubes		0.00	3633.75	3633.75	14.74	Sand	20701.07
Medium	MS	1346.56	290.70	1637.26	6.64	Sheet	5046.88
	Agar	650.39	0.00	650.39	2.64		
	Sucrose	444.36	255.82	700.18	2.84		
Hormones	BAP	43.76	0.00	43.76	0.18		
	IAA	3.83	0.00	3.83	0.02		
	NAA	0.00	0.36	0.36	0.00		
Electricity	Autoclave	631.20	181.69	812.89	3.30		
	Laminar	350.67	1094.38	1445.04	5.86		
	Incubation	4208.00	4845.00	9053.00	36.72		
Labor	Jar clean	37.89	0.00	37.89	0.15	Labor	1345.83
	Mprep	280.53	242.25	522.78	2.12		
	Mdisp	0.00	672.92	672.92	2.73		
	Culturing	1402.67	4037.50	5440.17	22.07		
Total cost (+ jars)		13437.36	15254.36	28691.72		Total cost (+ pot, sand)	50915.03
Total (- jars)		9399.86	15254.36	24654.22	100.00	Total cost (- pot, sand)	6392.71
Total shoots		121125		121125		Plants	121125
Cost per shoot (+jars)		0.11	0.13	0.24		Cost per plant (+ pot, sand)	0.42
Cost per shoot (- jars)		0.08	0.13	0.20		Cost per plant (- pot, sand)	0.05

Shoots were cultured at density of 1 shoots per 20 ml of agar solidified (7 g/l) full strength MS medium supplemented with sucrose at 30 g/l combination of BAP at 3.25 and IAA at 1.75 mg/l and the pH adjusted to 5.7. The cultures incubated for 75 days under constant temperature 25 ^{0} C and 16 hours of light and subcultured for four times.
Shoots rooted at density of 5 shoots per culture in 6 ml of liquid quarter strength MS medium enriched with sucrose at 20 g/l and NAA at 1.0 mg/l, pH 5.0 and the cultures incubated for 30 days under constant temperature and 16 hours of light.
Plantlets acclimatized by placing of 15 plantlets in 10’ pot filled of sand and placing each 6 pots inside polyethylene sheets under shade for 15 days. Polyethylene removed after 21 days and the plantlet kept under shade for 75 days
Ctubes (Culture tubes), Jclean (jars cleaning), Mprep (Medium preparation); Mdisp (Medium dispensing).
Culturing (separation and culturing of shoots under laminar)
Autoclave, Laminar and Incubation (Electricity cost of operating autoclave, laminar and incubation room)
RM (1 USA $= RM 3.25)

Table (4.53). Total cost and cost items of micropropagation stages (multiplication, rooting and acclimatization) of Moris pineapple staring by 12 shoots in Malaysian Ringgit (RM)

Cost items		Multiplication	Rooting	Total	%	Cost item	Acclimatization
C.tubes	Tubes	135.90	453.60	589.50	21.74	Pots	2973.60
Medium	MS	57.98	36.29	94.27	3.48	Sand	2584.11
	Sucrose	12.76	31.93	44.69	1.65	Sheet	42.00
	BAP	1.16	0.00	1.16	0.04		
	IBA	0.00	1.31	1.31	0.05		
Electricity	Autoclave	9.06	22.68	31.74	1.17		
	Laminar	39.00	136.00	175.00	6.45		
	Incubation	362.40	604.80	967.20	35.67		
Labor	Mprep.	12.08	30.24	42.32	1.56	Labor	672.00
	Mdisp	25.17	84.00	109.17	4.03		
	Culture	151.00	504.00	655.00	24.16		
Total cost		806.51	1904.85	2711.35	100.00	Total cost (+ pot, sand)	6271.71
Total shoots		15120	15120	15120		Total cost (- pot, sand)	714.00
Cost per shoot		0.05	0.13	0.18		Plantlets	15120
						Cost per plant (+ pot, sand)	0.41
						Cost per plant (- pot, sand)	0.05

Twelve shoots were cultured at density of 4 shoots per 8 ml of liquid full strength MS medium supplemented with sucrose at 20 g/l BAP at 2.0 mg/l and the pH adjusted to 5.0. The cultures incubated for 60 days under constant temperature 25 ^{0}C and 16 hours of light and subcultured for four times.
Shoots rooted at density of 5 shoots per culture in 6 ml of liquid quarter strength MS medium enriched with sucrose at 20 g/l and IBA at 2.0 mg/l, pH 5.0 and the cultures incubated for 30 days under constant temperature and 16 hours of light.
Plantlets acclimatized by placing of 15 plantlets in 10" pot filled of sand and placing each 6 pots inside polyethylene sheets under shade for 15 days. Polyethylene removed after 21 days and the plantlet kept under shade for 75 days.
Ctubes (Culture tubes), Jclean (jars cleaning), Mprep (Medium preparation); Mdisp (Medium dispensing).
Culturing (separation and culturing of shoots under laminar)
Autoclave, Laminar and Incubation (Electricity cost of operating autoclave, laminar and incubation room)
RM (1 USA $= RM 3.25)

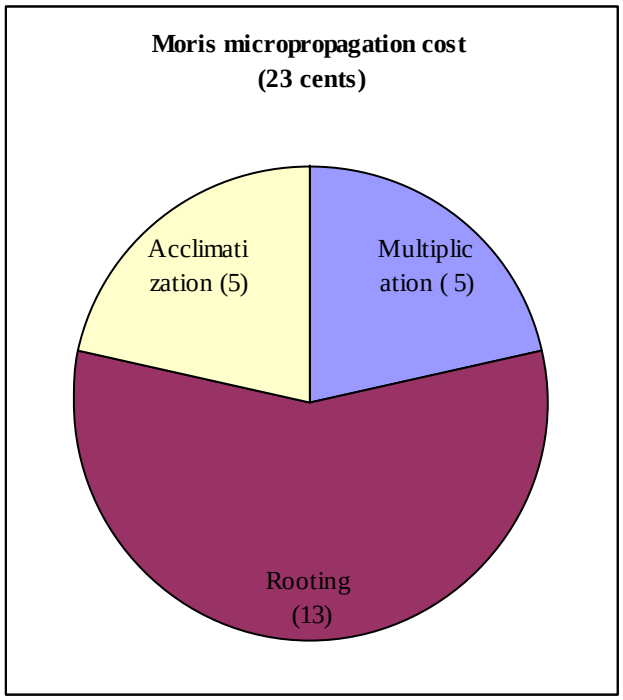


Figure (4.26). Cost of one propagule of Moris pineapple at different stages of propagule production

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