Optimizing of Root Induction in Oil Palm Plantlets for Acclimatization by Some Potent Plant Growt Regulators (PGRs)

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Abstract

Optimum root induction of vitro shoot of oil palm using various types and concentrations of Plant Growth Regulator (PGR) containing culturing media at 35 days after culturing was carryout. Forming root from shoot vitro were raised among three different culture media Murashige Skoog (1/2 MS, Strength MS and WPM). For rooting treatment in different NAA and PBZ concentration and culture medium were evaluated. Identical pattern occurred on WPM medium containing NAA and PBZ the highest mean number of root formation per explants was produced in treatment with 6 mg/l NAA and 9 mg/l PBZ with maximum rooting of 93 % was achieved. *Ex vitro* root induction were rooted when seradix#1 included in those experimental which expected result additionally rooted shoots were successfully acclimatized to soil. Approximately 80-100% of the *in vitro* raised plantlets with well developed shoot and roots were survived after transferring to mixed soil and grown well in the green house conditions. This suggested that the use of WPM medium supplemented with 6 mg/l NAA and 9 mg/l PBZ promoted root formation in oil palm effectively. In Rooted plantlets were acclimatized on medium composed of soil+ sand + and osmocote according to ratio (2: 2: 1).

Key words: Paclobutrazol, Woody Plant Media, Naphthalene acetic acid, PGRs, oil palm

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List of Abbreviation and Symbols

Bsh	: vascular bundle sheath
Cu	: cuticle
Cm	: centimeter
DNA	: deoxyribonucleic acid
EDS	: embryo derived shoots
FW	: fresh weight
gc	: guard cell
g	: gram
mg/l	: milligram perlitre
ml	: milliliter
MS	: Murashige and Skoog (1962)
NAA	: 1-naphtaleneacetic acid
Ol	: outer ledge
Pal	: palisade cell
PBZ	: paclobutrazol
PGRs	: plant Growth Regulators
Ph	: phloem
p	: probability level
SD	: standard deviation
SE	: somatic embryogenesis
Spo	: spongy parenchyma
St	: stomata
USDA	: United State Development Agriculture
WPM	: woody plant medium
X	: xylem
%	: percentage

Chapter 1
Introduction

The fast booming of world market demand, development of oil palm has been expanded tremendously in recent years as a major source of the world supply of oil and fats. Recently, Southeast Asia is the dominant region of production with Indonesia being the leading producer and exporter of palm oil (USDA, 2007).

Oil palm is an arborescent monocotyledon which cannot be multiplied by conventional means of vegetative propagation. Success in plant regeneration by means of somatic embryogenesis has already been reported on many species of palms (Khaw and Ng, 1998). The establishment of plant regeneration in oil palm by somatic embryogenesis is satisfactory. Micropropagation has also been identified as suitable alternative for rapid or large-scale plant production (Rabechault *et al.,* 1974). However, several authors reported *in vitro* multiplication of oil palm in associated with difficulties in different stages of micropropagation particularly high-frequency rooting of microshoots remains a failness of roots formation (Kanchanapoom and Domyoas, 1999). The oil palm as monocotyledon is growing from one primary shoot meristems and has no obvious morphological base for vegetative propagation. Cloning of oil palm is performed by somatic embryogenesis on calli of leaf origin according the methods of CIRAD-CP in France which was developed by Pennetier *et al*. (1981). The origin source of explants is generally young leaves.

In this recent era, *in vitro* propagation has revolutionized commercial nursery business (Pierik, 1991). Significant feature of *in vitro* propagation procedure is an enormous multiplicative capacity in a relatively short span of time, production of health and disease free plant and its ability to generate propagules around the year (Dhawan, 1986). Plant tissue culture is the science growing plant cells, tissue or organs isolated from the mother plant, on artificial media. One of prior thing to the success of micropropagation is the very high mortality rate of the vitro plantlets either during acclimatization phase or during transfer under field conditions. Generally, most of the *in vitro* derived plantlets fail to survive when they were transferred to field conditions. It has

been estimated that only 25 percent of the *in vitro* regenerated plantlets has been successfully transplanted *ex vitro* and still fewer can be adapted to the field conditions. Such a disappointing state of affairs has been attributed to certain underlying causes, of which the aberrant features of *in vitro* derived plantlets are significant (Schultz, 2001). Several problems were faced among which the transfer of *in vitro* plantlets to *ex vitro* conditions. The most critical limit the widespread use of micropropagation for many species losses from 50 to 90% of *in vitro* propagated plantlets have occurred when transferring them to the soil (Sutter, 1985). Consequently, the transplantation stage continues to be a major bottleneck in the micropropagation of many plants. Plantlets that have grown *in vitro* have been continuously exposed to a unique micro-environment that has been selected to provide minimal stress and optimum conditions for plant multiplication. Plantlets were developed within the culture vessels under low level of light, aseptic conditions, on a medium containing simple sugar and nutrients to allow for heterotrophic growth and in atmosphere with high level of humidity make them survive at low frequency of survival just after transfer to soil (Kadlecek *et al*., 2001). To improve survival rate of the vitro plant to soil or field conditions the use of chemical such as plant growth regulators are of great interested. Growth regulators have been widely used to improve the quality of vitro-plant. Optimization of growth regulator related to enhance chlorophyll and carotenoid content of leaf and stimulated or regulated the action on biosynthesis of chloroplast pigments (Iqtidar *et al.,* 1994). The ability of PBZ to induce root from vitro-plant has not been reported. It is effect on root induction of vitro-shoot of oil palm is described in this report. The advantages will be very important in commercial propagation of oil palm through tissue of culture technique. Moreover, recent development of highly active growth retardants has further enhanced the potential uses of chemical regulators. Among them, paclobutrazol (PBZ) is widely used (Fernandes *et al.,* 2004). However, substantial information on *in vitro* rooting and acclimatization of oil palm plantlets to soil has been rare up to now. Therefore, the objective of this study was to develop a protocol for rooting and acclimatization of the oil palm plantlets by using the combination of PBZ and NAA. Moreover, we also report the successful effect of PBZ

on enhancing transplanting to soil and particularly high frequency rooting of microshoots of oil palm.

1.1 Objectives

1. to enhance root induction of vitro-shoots of oil palm by some potent PGRs both *in vitro* and optimize survival rate in *ex vitro* conditions.
2. to study anatomical characters of root and leaf developed from different PGRs containing medium.
3. to acclimatize *vitro* plant to field conditions in order to minimize the failure during transplanting.

1.2 Literature Review

1.2.1 *In Vitro* Plant Proliferation

Tissue culture displays many advantages such as rapid propagation of plants, massive production of disease and virus free seedlings and preservation of plant species resources. Commercial propagation of oil palm through tissue culture technique is widely used (Khaw and Ng, 1998, 1999). Long term culture is unavoidable to induce callus and embryogenic callus. Usually, it takes 3-6 months to induce callus and 3-9 months to induce embryogenic callus (Khaw and Ng, 1999). Moreover, plant tissue culture can be used for the mass propagation of other plants. It may be argued that such a method could be a process to revolutionize some aspect of horticulture and agriculture. The production of millions of plants via suspension culture and embryogenesis can be achieved (Reinert and Bajaj, 1977).

1.2.2 Plant Growth Regulators (PGRs)

1.2.2.1 Paclobutrazol (PBZ)

Paclobutrazol (PBZ) ([2R, 3R + 2S, 3S) – 1 – (4-Chloro-phenyl) 4,4-dimethyl-2-(1,2,4-triazol-l-yl)-Pentan-3-ol]) are a triazole type plant growth retardant which blocks gibberellins biosynthesis and involved in reducing abscisic acid, ethylene and indole-3-acetic acid while increasing cytokinin levels (Anonymous, 2008). PBZ treated plant have dark green foliage. This has been associated with the increment in chlorophyll content in leaf tissue (Sopher *et al*., 1999; Berova and Zlatev, 2000; Sebastian, 2002). More densely packed of chlorophyll content may be ascribed to higher cytokinin content that stimulated chlorophyll biosynthesis and reduced chlorophyll catabolism (Berova and Zlativ, 2000; Sopher *et al.,* 1999; Davis *et al*., 1988; Joseph and Yelenosky, 1997). There are reports indicating that PBZ enhances photosynthetic efficiency in plant because of the higher chlorophyll content. Stimulating the rate of photosynthesis enhances rate of survival on *ex vitro* during transplanted to field. Moreover, PBZ increases the survival rate of plant under field condition by modification a number of physiological responses. A reduction in the rate of transpiration (due to reduction in leaf area), increased diffusive

resistance, reduction in water potential, increase relative water content, less water use, and increased oxidant activity (Marshal *et al.,*1991; Elliasson *et al*., 1994).

Rooting of micro-shoots is critical phase in plant micropropagation systems of woody plant. Many researchers have demonstrated that the initiation of adventitious roots *in vitro* in excised shoots of the oil palm is related to several factors. The most important factors in rooting induction or initiation are concentration of auxin and treatment duration (Naija *et al.,* 2008). PGRs are among the external stimuli most often employed to induce somatic embryo (SE) and to regulate the further development of embryogenic tissue, whether PGRs act not only as stimuli but also involved directly in the mechanisms that regulate gene expression (Gaspar *et al.,* 2003). PGRs is critical factor in growth and development of plant tissue, it also interact with important biological determinants. Moreover, genotype and physiological state of plant will determine the morphological response. Media are optimized for each genotype and explants. Generally, initial concentration of PGRs in tissue is low and gradually increases with time due to increment rate of uptake. Auxin, a plant hormones, is a growth regulator that can promote *in vivo* cell elongation and division.

1.2.2.2 Napthaleneacetic acid (NAA)

α– Napthaleneacetic acid (NAA), a synthetic auxin, is widely used in horticulture to stimulate the formation of adventitious roots. In most cases, high levels of NAA in tissue are correlated with the process of adventitious rooting (Sagee *et al.,* 1992). In the rooting experiments of *Gerbera jamesonii* B., the shoots of more than one cm length were cultured on ½ MS medium to which 2,4-D and NAA were added at 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg per litre concentrations (Karnataka, 2008). Study of treatments on the rooting performance of *Juglans regia* L. was employed by Saadat and Hennerty (2001) who revealed that root development was done in rooting media containing IBA and NAA (2, 3, 4 and 5 mg/l) in darkness. On the other hand, sucrose as energy sources play an important role in *in vitro* cultures as an energy and carbon source, as well as an osmotic agent. In addition, carbohydrate-modulated gene

expression in plants is known (Koch, 1996). It is now clear that sugars, such as sucrose generally though to be an energy providing roles in term of interaction with hormones or their transduction chains.

In this present study, the results from experiments on the influence of PBZ added to the rooting medium for vitro shoot of oil palm on the root related to acclimatization, and on the growth and development of plants in the greenhouse will be described. However, both rooting and acclimatization procedures need to be improved for mass propagation of oil palm.

1.2.2.3 Seradix

Seradix# 1, 4-indol-3-ylbutyric acid, is rooting hormone with concentration (%) of 0.1% w/w and appearances pink colored fine powder (Anonymous, 2009). Nawrocka and GrzeskowiakRoot (2004) revealed that system size and the percentage of rooted cuttings depended on the applied growth substance as well on species. The highest rooting level (100%) was recorded for *Rhododendron* Cunningham's White under influence of 4% IBA and Seradix B2. Moreover, Khan *et al*. (2004) reported that seradix resulted in high rooting percentage, development and mortality was 2.33% as compared to control and different levels of IBA in *Rosa bourboniana*. The highest rooting percentage of cuttings of the Galega vulgar clones under study was obtained with Seradix 14.6% (powder formulation) (Serano and Serano, 2002). According to Rozihawati *et al.* (2000) application of rooting hormone Seradix#1 is required to get good rooting 100% of *Labisia pumila* cuttings.

Chapter 2
Materials and Methods

2.1 Plant material

Plant material used in this research study was embryo derived shoots (EDS). Those shoots were derived *in vitro* from the mature trees taken from Laboratory of Crop Biotechnology, Department of Plant Science, Faculty of Natural Resources, Prince of Songkla University Thailand. According to the previous protocol reported by Te-Chato (unpublished data). The EDS were subcultured monthly interval for three months in MS medium supplemented with a low concentration of NAA, 3% sucrose under light condition.

2.1.1 EDS preparation and treatments

Individual vitro-shoot was excised, removed from shoot clumps and transferred to three different culture medium components (1/2 MS, MS and WPM) incorporated with different concentration of NAA (0, 2, 4, 6, 8 mg/l) and PBZ (0, 3, 6, 9, 12 mg/l). Three different culture media together with those combinations of PGRs were shown in Table 1.

2.1.2 Culture media

The culture media used in this experiment were basal MS, half-strength MS and WPM. The details of components in each culture media were shown in appendix 1. Each culture medium was supplemented with 7.2% sucrose, various concentrations of NAA and PBZ. All culture media were adjusted pH to 5.8 before adding agar-agar at 0.6% and autoclaved at 120^{o}C at 1.07 kg/cm^{2} for 15 min. EDS was raised on those culture media under *in vitro* conditions. The elements necessary for the growth of whole plants in this study included various vitamins, plant growth (paclobutrazol, NAA) and carbon sources (sucrose with higher concentration 7.2%). Those elements, as well as growth regulators frequently altered morphogenesis in plant tissue.

In case of culturing on solid media, they were cultured in medium to large bottle containing 20 ml of culture medium under static condition. For culturing in liquid medium agar-agar was removed and the cultures carried out in 125 ml Erlenmeyer flask containing 30 ml of culture medium.

Table 1 Various culture medium component in combination with PGRs used for induction root from *vitro* shoot of oil palm

Culture Media	PGRs (mg/l)	
	NAA	PBZ
½ MS	0	0
	2	3
	4	6
	6	9
	8	12
MS	0	0
	2	3
	4	6
	6	9
	8	12
WPM	0	0
	2	3
	4	6
	6	9
	8	12

Twenty shoots were used for each treatment.

2.2 Effect of some potent PGRs on shoot and root induction *in vitro*

Two factors; culture media and PGRs were investigated for root induction. Three kind of culture media; ½ MS, MS and WPM were used. For MS and WPM (as shown in Table 1) they were supplemented with 7.2% sucrose whereas ½MS was supplemented with 3% sucrose. Each culture medium component was supplemented with NAA (0, 2, 4, 6 and 8 mg/l) and PBZ (0, 3, 6, 9 and 12 mg/l). All media were adjusted pH to 5.7 before autoclaving and solidified with 0.6% agar. The cultures were maintained under 14 h photoperiod of 25 µmol/m^2/s at 28±1^{o}C. After culturing for 4-6 weeks multiple shoot formation, percentage of shoot produced root, number of roots and root length were recorded and statistically compared in each factor separately using completely randomized design (CRD). Twenty shoots were used for each treatment.

Complete plantlets (shoots with roots) obtained from the above procedures were gently excised from test tube and agar was absolutely removed. The plantlets are placed in 4 inch black polybag containing soil mixture and kept under high humidity and low light intensity. During 4 weeks of acclimatization under gradually decrease in humidity and increase in light intensity. Then survival rate of the plants derived from each culture medium components and PGRs was recorded and compared statistically using CRD.

2.3 Effect of Seradix and PBZ on root induction *ex vitro*

Vitro-shoots of oil palm without root obtained from somatic embryos were excised from shoot clump in test tube and agar was absolutely removed. Those shoots were passed through two different dipping with two PGRs; Seradix# 1 (commercial grade of IAA) and PBZ. In case of PBZ, the best concentration obtained from *in vitro* experiment was applied. PGR dipped shoots were immediately placed in 4 inch black polybag containing soil mixture and kept in greenhouse under 50% shading. The moister was kept at 100% during 4 weeks by covering the shoot with glass bottle subsequent to gradually open to greenhouse conditions. At this stage, survival

percentage of complete plantlets obtained from each PGR was recorded and compared statistically using CRD. Twenty shoots were used for each treatment.

2.4 Anatomical study of leaf and root

After three months of culture, leaves and roots from complete plantlet *in vitro* was collected. At least three representative plants per treatment were used in this experiment. Sampling was carried out according to the following procedure. Leaf and root samples were sliced in thin section using sharp lazor blade. Those segments are placed on glass slide with a drop of distilled water. The slides were then covered with cover slip. The observed was carried out under compound microscopy. Anatomical characteristics of cells in each layer were recorded and compared among the plants or samples in different culture media and PGRs.

Chapter 3
Result

3.1 Effect of some potent PGRs on shoot and root induction *in vitro*

3.1.1 Multiple shoot formation

Among culture media tested WPM gave the best results in all parameters observed, multiple shoot formation, growth of shoot, leaf and root. So, the following results demonstrated the effects of WPM together with responding PGRs. Shoots cultured on WPM supplemented with 6.0 mg/l NAA and 9.0 mg/l PBZ provided a new forming shoot. Approximately two shoots were obtained after culture for 6 weeks. Physically, new shoot grew vigorously while they were maintained on WPM medium. During the first 4 weeks of cultures, the external leaf sheaths of shoot, which initially white, turned green, swelled up and then opened slowly at the tip, then younger leaf primordial grew out (Figure 1).

All concentrations of NAA and PBZ containing WPM were effective for inducing number of multiple shoot formation. NAA at 6 mg/l alone or in combination with 3, 6 and 9 mg/l PBZ resulted in multiple shoot formation. For growth rate of those shoots in term of shoot length and leaf width there were significant different ($p<0.01$). NAA at 6 mg/l in combination with 9 mg/l PBZ gave the highest results in those parameters (Table 2).

Increase in concentration of NAA and PBZ tended to increase the number of multiple shoot formation, optimize the caused show growth of the shoot. NAA at higher then 6 mg/l in combination with PBZ at higher then 9 mg/l inhibited of the shoot.

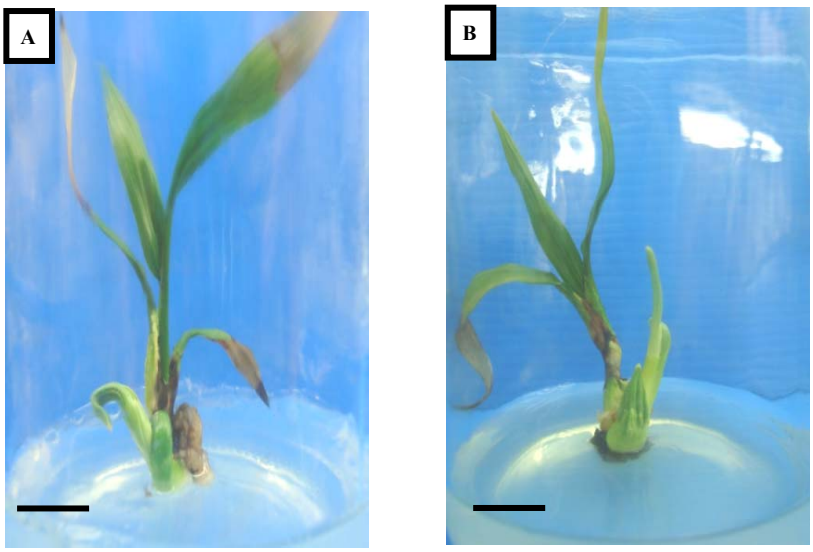

Figure 1 New forming shoot arose from shoot apex in WPM in combination with 6 mg/l NAA and 9 mg/l PBZ after 6 weeks of culture (A) and after 7 weeks of culture (B). (bar=0.33 cm)

Table 2 Effect of WPM medium supplemented with NAA and PBZ at different shoot growth and proliferation after 6 weeks of culture.

PGRs		Leaf width	Shoot length	Avg. no. of
NAA (mg/l)	PBZ (mg/l)		(cm)	shoot
6	0	0.90 abc	9.13 bc	0.13
	3	1.00 ab	9.66 b	0.16
	6	0.86 abc	8.50 bcd	0.06
	9	1.20 a	11.4 a	0.20
	12	0.83 abc	8.26 cd	0.00
F-test		**	**	Ns
C. V. (%)		27.7	8.6	55.1

** significant different at $p<0.01$

Means within a column followed by common letter do not differ significantly by DMRT.

Multiple shoot formation was not obtained in higher concentration of PBZ (12 mg/l) containing WPM. These chemical retardant were extremely inhibited shoot initiation and growth was also limited (Table 2).

3.1.2 Shoot Length

WPM supplemented with all combinations of NAA and PBZ promoted the greatest result in shoot length among three different culture media tested. In the absence of PBZ shoot length was significant lower than PBZ containing medium (Figure 2). PBZ at concentration of 9 mg/l in combination with 6 mg/l NAA containing medium promoted the highest shoot length at 11.4 cm, significant difference to other concentrations (Figure 2). From the observation it was also revealed that shoots grew rapidly and vigorously within 3 moths of culture.

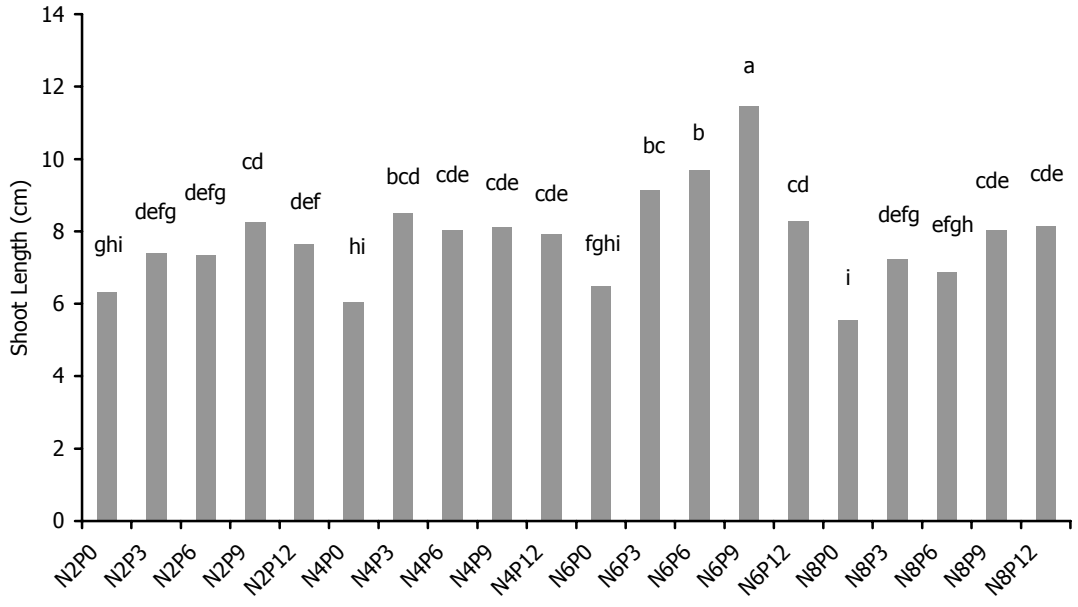

Figure 2 Effect of various combinations of NAA and PBZ containing WPM on shoot length after 8 weeks of culture.

Mean within histogram followed by common letter do not differ significantly by DMRT.

3.1.3 Stem Width

The stems obtained from PBZ containing were thicker than control treatment (WPM without PBZ). The highest stem width was obtained from 6 mg/l NAA and 9 mg/l PBZ containing WPM Without PBZ stem width decreased tremendously. Significant difference was not observed among the combinations NAA and PBZ (Table 3). However, low concentration of NAA (2-4 mg/l) alone (without PBZ) in WPM gave significant result in width of stem (Figure 3). In addition, color of stems changed from normal green to dark green indicating that the shoots were healthy (Figure 4).

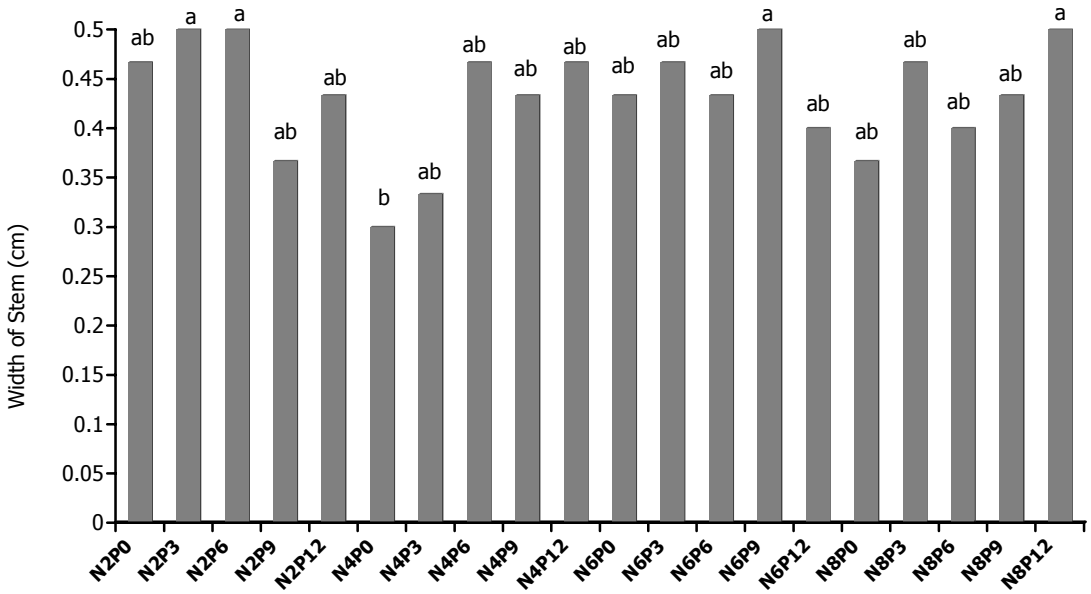

Figure 3 Effect of various combinations of NAA and PBZ containing WPM on stem width after 8 weeks of culture.
Mean within histogram followed by common letter do not differ significantly by DMRT.

Table 3 Effect of various concentration of NAA and PBZ containing WPM on some morphology of oil palm plantlets after 6 weeks of culture.

PGRs		Stem width	Number of leaves
NAA (mg/l)	PBZ (mg/l)		
6	0	0.43	3.6 dc
	3	0.46	4.0 bcd
	6	0.43	5.0 ab
	9	0.50	5.7 a
	12	0.43	5.0 ab
F-test		ns	**
C. V. (%)		19.8	20.9

ns: not significant difference **: significant difference at $p<0.01$

Means within a column followed by the same letter in common do not differ significantly by DMRT.

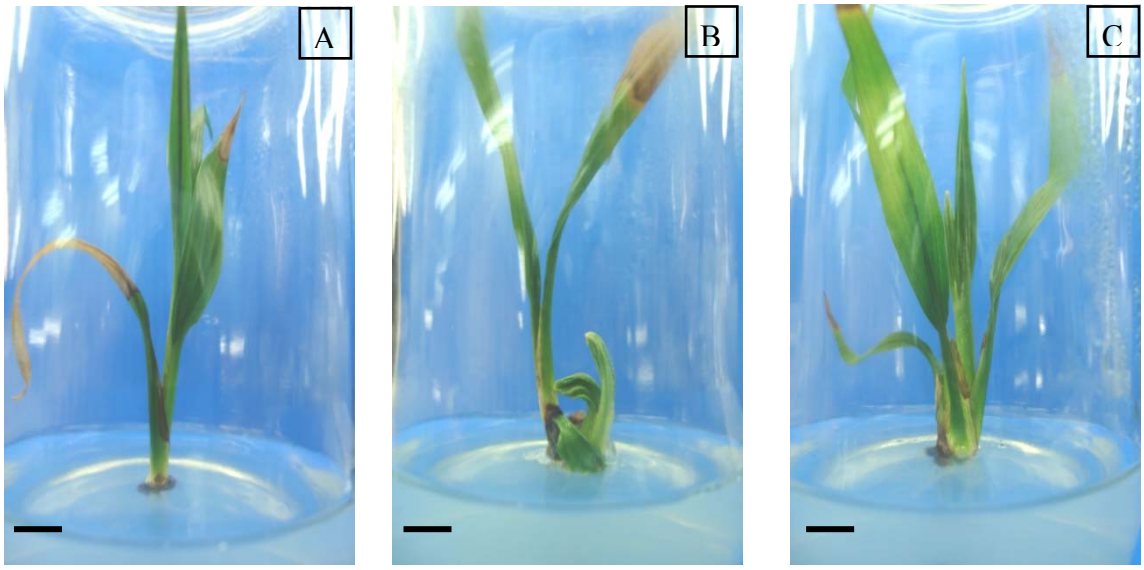

Figure 4 Width of stem or bolt in different culture media supplemented with NAA and PBZ after 6 weeks of culture (bar=0.33 cm)

A: ½ MS B: MS C:WPM.

3.1.4 Leaf Number

The development of leaves was promoted by the increment concentration of PBZ and NAA. This positive effect on leaf number became significant difference after six weeks after culture. At the end of the experimental period, calculation of the total number of leaves which had developed during 6 weeks confirmed the significant effect of various concentrations of NAA and PBZ (Figure 5). The optimum concentration of NAA and PBZ to gave the highest result in leaf number was 6 and 9 mg/l, respectively. NAA at lower or higher than this concentration in combination with all concentrations of PBZ gave the lower result significantly (Table 3, Figure 5).

Emergence of the new forming leaf developed after 2 weeks of culture, but significant differences was not observed. Significant difference was observed after 6 weeks of culture. The highest number of leaves was obtained from WPM media supplemented with 6 mg/l NAA and 9 mg/l PBZ at 5.7 leaves/shoot.

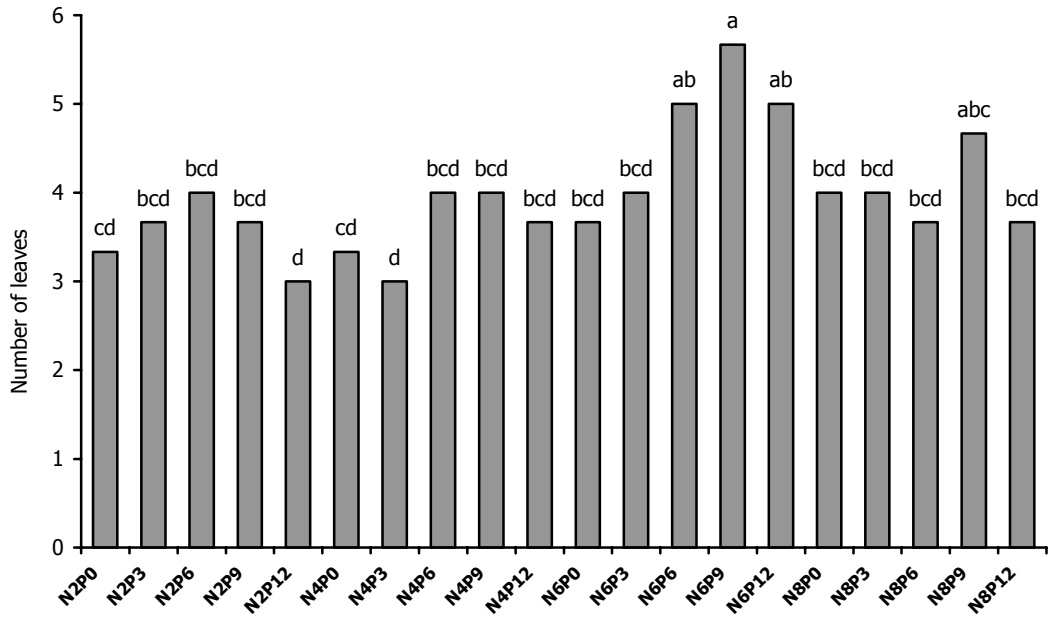

Figure 5 Effect of various combinations of NAA and PBZ containing WPM on number of leaves after 8 weeks of culture.

Mean within histogram followed by common letter do not differ significantly by DMRT.

3.1.5. Leaf Width

PBZ promoted leaf width in the presence of NAA, especially NAA at concentration of 6 mg/l. At this concentration of NAA the increment of PBZ increased leaf width significant different to other combinations (Figure 6). An optimum mean leaves width was 1.20 cm obtained from WPM medium supplemented with 6 mg/l NAA and 9 mg/l PBZ. Leaves of the PBZ-treated shoots were relatively wider than those of the non treated shoots. As consequence, the treated shoots developed a more compact and perfect shape.

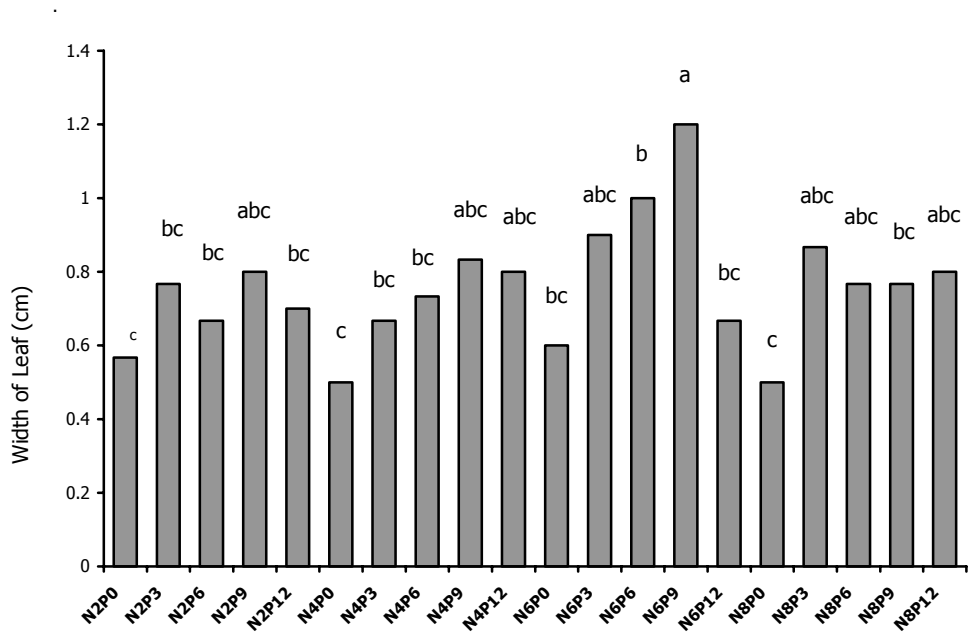

Figure 6 Effect of various combinations of NAA and PBZ containing WPM medium on leaf width after 8 weeks of culture.

Mean within histogram followed by common letter do not differ significantly by DMRT.

3.1.6 Root Formation

In vitro shoot of oil palm were able to form root when cultured on WPM media supplemented with various concentration of NAA and PBZ. The greatest rooting

percentage was obtained on WPM medium supplemented with 6 mg/l of NAA and 9 mg/l PBZ at 93% (Figure 7).

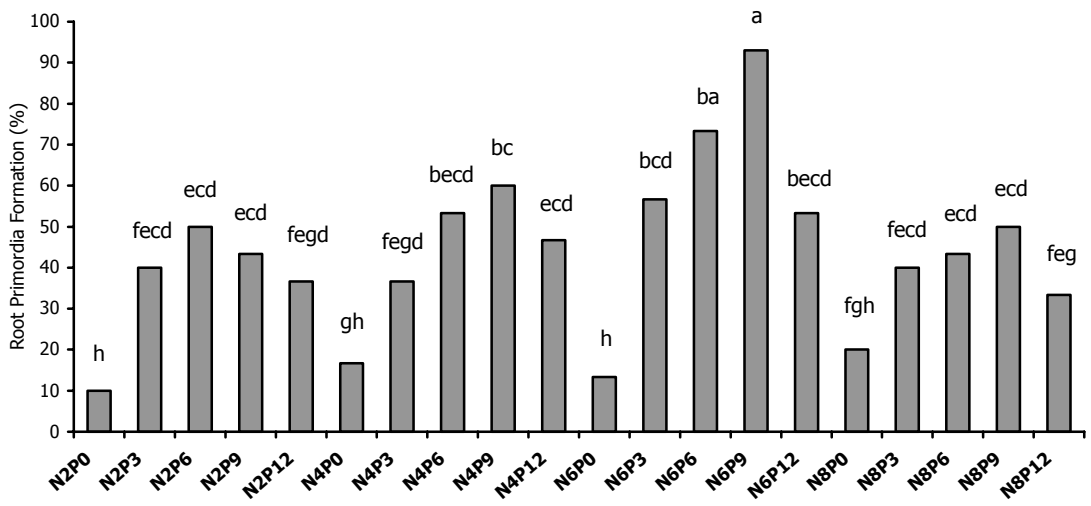

Figure 7 Effect of various combinations of NAA and PBZ containing WPM on root induction after 8 weeks of culture.

Mean within histogram followed by common letter do not differ significantly by DMRT.

The average number of root induced from each shoot and their length increased as the concentration NAA and PBZ was increased. The most effective concentration of NAA and PBZ was 6 and 9 mg/l, respectively. The maximum percentage of root formation obtained from these PGR containing medium was nearly 90% significant to other combinations of NAA and PBZ (Figure 7). The lowest result in root formation was clearly seen in the absence of PBZ. Optimum concentration of both NAA and PBZ also gave vigorous and healthy root with an extensive growth. The appearance of all roots produced in PBZ containing WPM was thick and stumpy, especially a high concentration of NAA and PBZ (8-12 mg/l) (Figure 8, 9). Our observation indicated that NAA and PBZ at optimum concentration containing WPM medium with high concentration of sucrose (7.2%) gave rooting percentage better than normal concentration of sucrose (3%).

Figure 8 Root development from shoot *in vitro* on WPM supplemented with NAA and PBZ.

A: cluster of EDS

B: excised single shoot

C: emergence of root on WPM with 6 mg/l NAA and 9 mg/l PBZ after 5 weeks of culture.

D: emergence of root on WPM with 6 mg/l NAA and 9 mg/l PBZ after 7 weeks of culture.

Rapid growth of root was obtained on WPM medium supplemented with PBZ and NAA in the presence of high concentration of sucrose (7.2%). It indicated that NAA and PBZ in WPM medium was able to induce root formation of vitro-shoot of oil palm, which roots were completely or normally form (Figure 9).

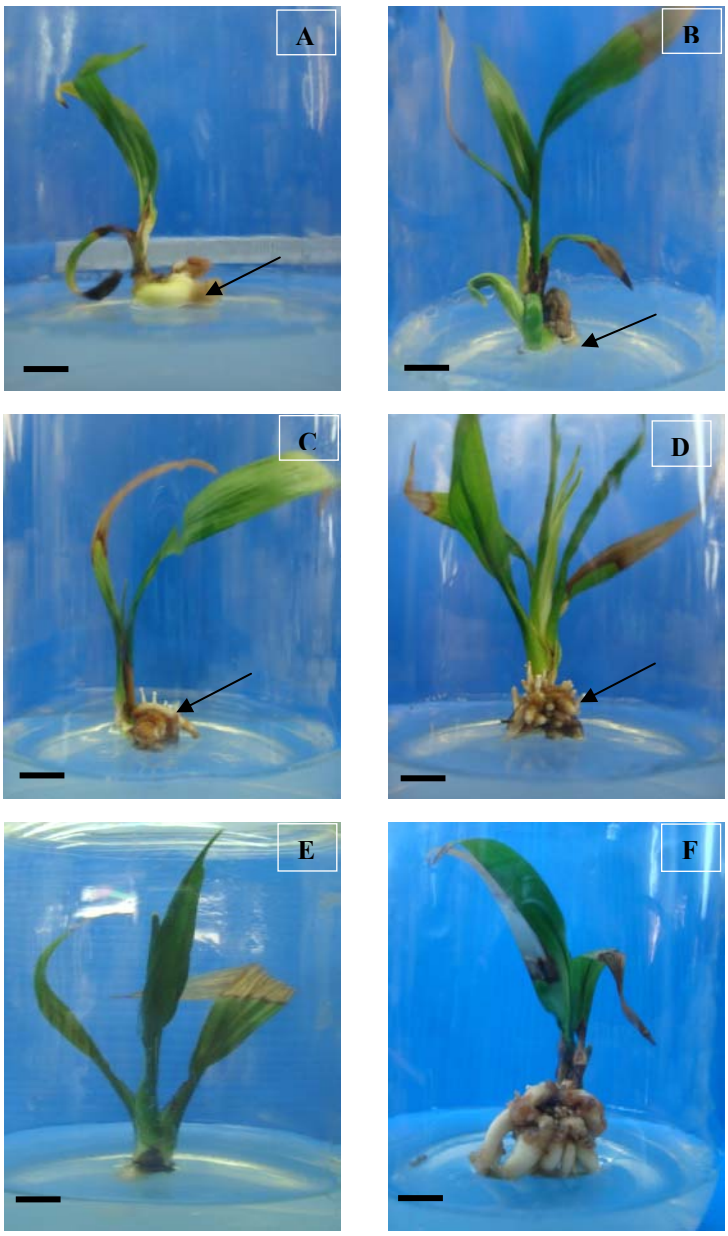

Figure 9 Root formation (arrow) from vitro-shoot *in vitro* of oil palm on WPM supplemented with NAA and PBZ after various time of culture. (bar= 0.33cm)

A: 5 weeks in 6 mg/l NAA, 9 mg/l PBZ

B: 6 weeks in 6 mg/l NAA, 9 mg/l PBZ

C: 7 weeks in 6 mg/l NAA, 9 mg/l PBZ

D: 8 weeks in 6 mg/l NAA, 9 mg/l PBZ

E: controlled treatment

F: 8 weeks in 6 mg/l NAA, 8-12 mg/l PBZ

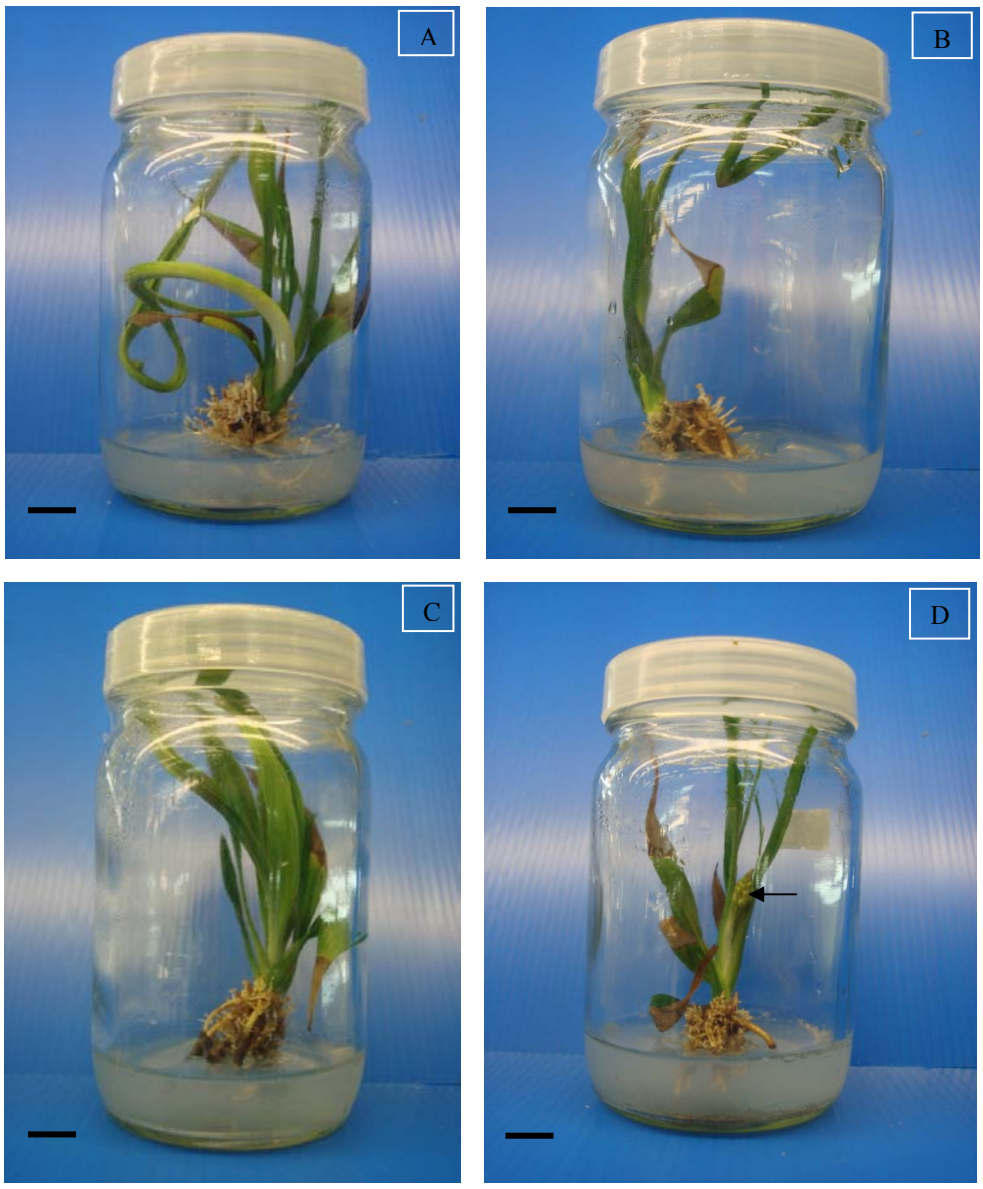

Figure 10 Root formation under treated by NAA and PBZ in WPM after 3 months of cultured. (bar = 0.45cm)

A: Number of secondary roots and vigorous shoot.

B: Formation of primary root with lateral roots.

C. Abundant of root formation and large stem in 6 mg/l NAA and 9 mg/l PBZ

D. Floral organ development (arrow) in 12 mg/l PBZ and 6mg/l NAA containing WPM.

3.1.7 Number of root

Roots began to emerge on 7 weeks and by week 8 were clearly visible. The number of roots was determined on week 8 and again on week 9 and the results were shown in Figure 11.

Rooting was induced over the entire range of NAA and PBZ concentration tested. High root numbers induced by medium supplemented by NAA and PBZ. Shoots explants cultured on low concentration (2-4 mg/l NAA and 3-6 mg/l PBZ) of both PGR yielded a greater number of roots than higher concentrations (8 mg/l NAA and 12 mg/l PBZ).

The great number of root formation was (6.67/shoot) obtained from WPM as rooting media containing 6 mg/l NAA and 9 mg/l PBZ. As far as the number of roots was concerned, there were significant different among culture media and PGRs (Figure 11). The plants, which were grown on medium with 6 mg/l NAA in combination with 9mg/l PBZ had the highest average number of roots per plant. Culture media enriched with paclobutrazol and NAA gave higher root number produced significantly more than non-treated shoots. With more roots formed from vitro-shoot it is markably that PGRs affected on stimulation of root induction.

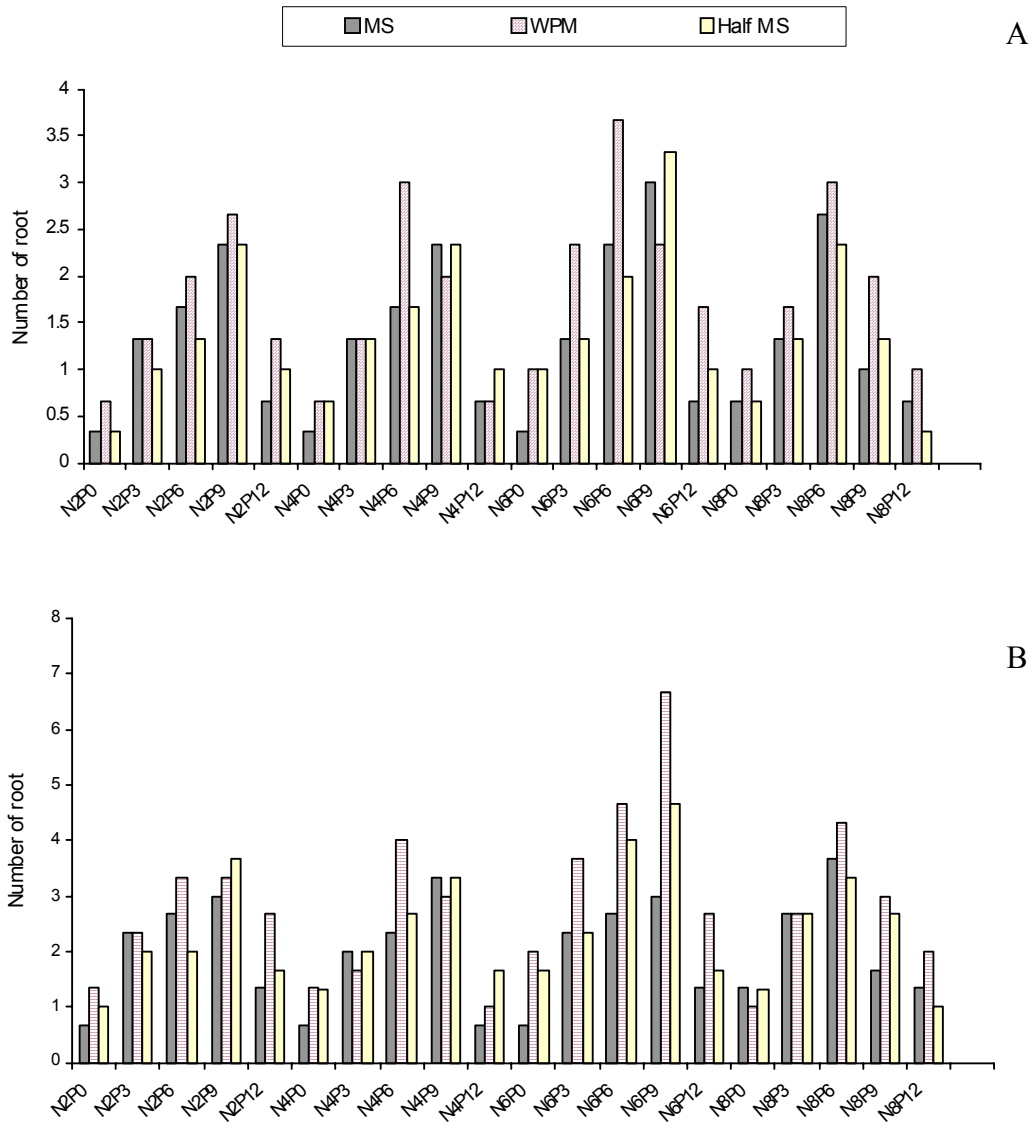

Figure 11 Effect of various combinations of NAA and PBZ containing different culture media on number of roots after 7 (A) and 9 weeks of cultured (B).

3.1.8 Root length

Among three different culture media tested WPM gave the best result in root length (Figure12). Significant difference ($p<0.01$) was observed when NAA at 6 mg/l was used in combination with PBZ at concentration ranging from 6 to 9 mg/l. This culture medium in the presence of PBZ enhanced root growth. The longest roots (3.66 cm) were found on the medium containing 6 mg/l NAA incombination with 9 mg/l paclobutrazol while the shortest ones (2.00 cm) were obtained from medium without PGR (Table 4).

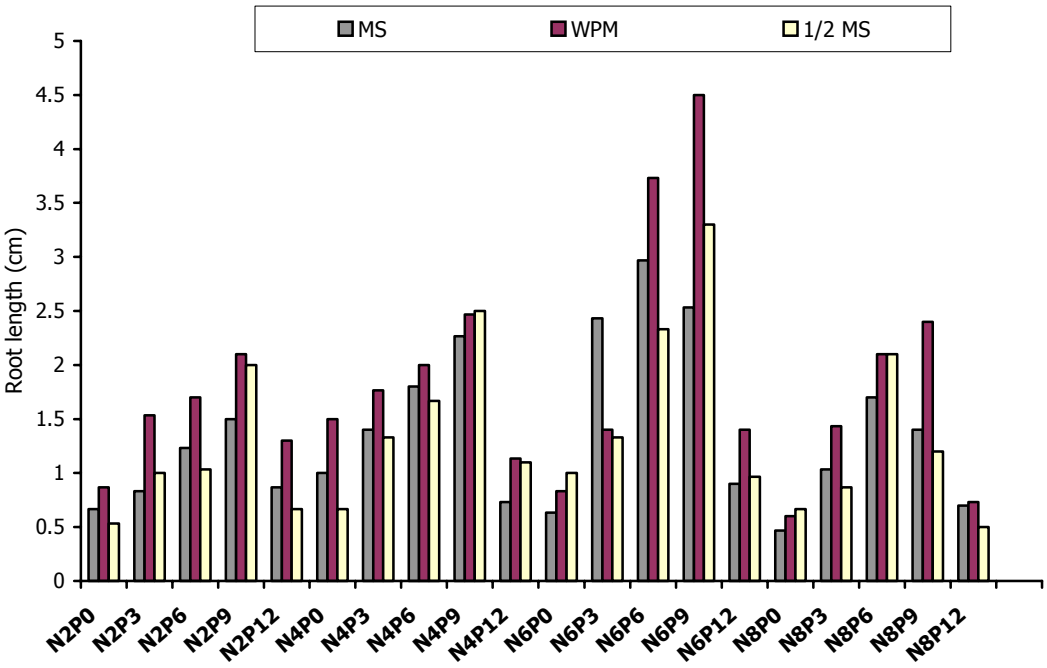

Figure 12 Effect of various combinations of NAA and PBZ containing different culture media on root length after 7 weeks of culture.

Table 4. Effect of various combinations of NAA and PBZ containing WPM on root length and number of root from vitro-shoot after 7 weeks of culture.

PGRs		Root length (cm)	Root number
NAA (mg/l)	PBZ (mg/l)		
6	0	2.00 bcde	3.67 bcde
	3	2.67 abc	4.33 bc
	6	3.00 ab	4.67 b
	9	3.66 a	6.67 a
	12	2.33 bc	4.00 bcd
F-test		**	**
C. V. (%)		37.14	38.59

** significant different at $p<0.01$

Means within a column followed by the same letter do not differ significantly by DMRT.

The fantastic percentage of root development started from first week to the eight weeks of cultured. Among three different rooting media WPM gave the best result in root induction (nearly 80%) followed by MS (30%) and 1/2MS (20%) (Figure 13).

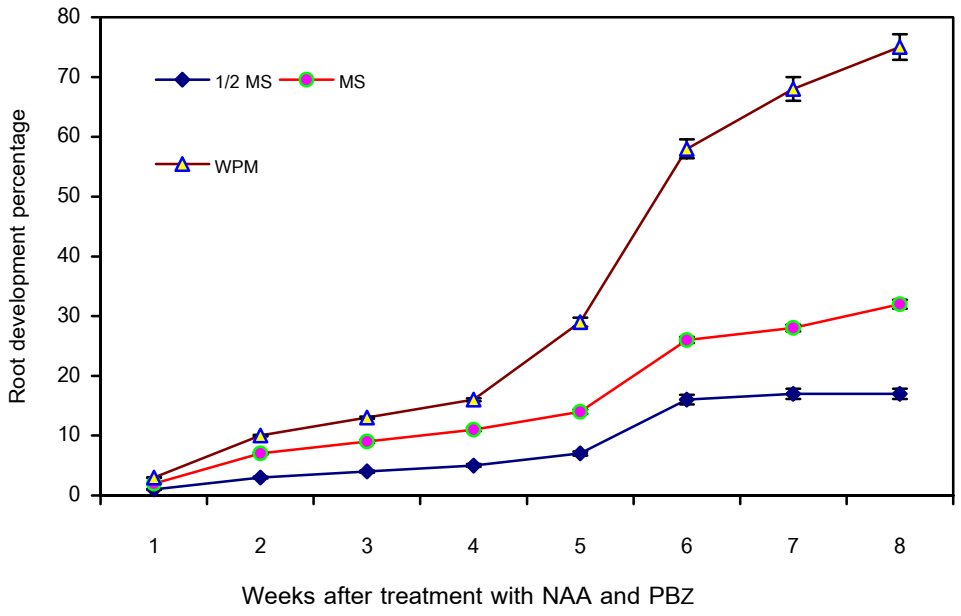

Figure 13 Effect of rooting media supplemented with 6 mg/l NAA and 9 mg/l PBZ on root percentage development of vitro shoot of oil palm.

3.1.9 Chlorophyll content

The chlorophyll content of the leaves increased with the age of the plant. All combinations NAA and PBZ containing WPM increased the chlorophyll content when compared to control (Figure. 14). Increase in concentration of either NAA or PBZ promoted the increment of chlorophyll content significant different ($p<0.01$) to lower concentration. The highest total chlorophyll content was obtained from 6 mg/l NAA and 9 mg/l PBZ at 3.54 mg/gFW. The lowest total chlorophyll contents were found on control treatment at 1.94 mg/gFW. Moreover, NAA at concentration higher than 6 mg/l (8 mg/l) alone or in combination with all concentrations of PBA caused the decrement in chlorophyll content (Figure 14).

Besides, the highest of chlorophyll content was observed on medium WPM supplemented with 7.2% sucrose. This high concentration of sugar was superior to photosynthesis in general and corresponded with stomata resistance as well.

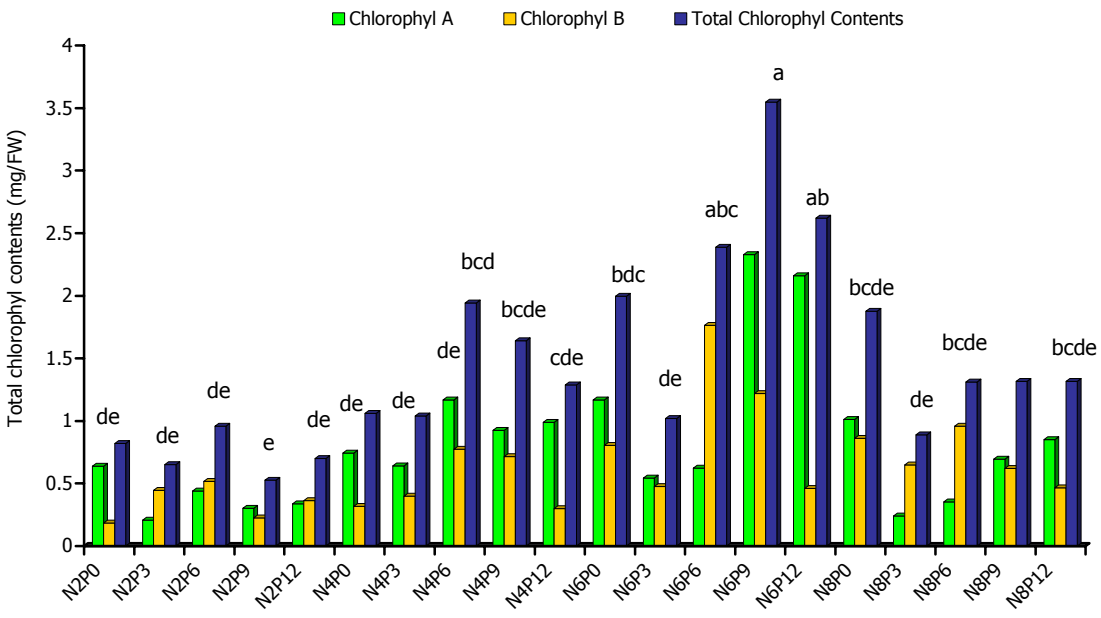

Figure 14 Effect of various combinations of NAA and PBZ containing WPM on chlorophyll content after 8 weeks of culture.

Mean within histogram followed by common letter do not differ significantly by DMRT.

3.2 Effect of Seradix and PBZ on *ex vitro* rooting

Generally, Seradix was used as rooting hormone for cutting of flowering and ornamental plants. Powder of this chemical substance was directly dipped with the cut end of soft or semi-soft wood, then inserted into pot. For PBZ there have no reports on root induction. But it caused water stress and might effective in inducing root. So, this experiment was set up to investigate the mutual effect of the two chemical substances. The results showed that fine roots were achieved within 3 months of transfer to soil. PBZ at concentration of 6 mg/l with 1mg/l Seradix# 1 gave the best result in root induction *ex vitro*. Additional of various concentrations of PBZ in combination with Seradix# 1 tended to assists adventitious root formation as shown in (Figure. 15). However, the maximum numbers of root could be produced and hardened enough to transfer for plantation within 3 months. Application of PBZ *ex vitro*: condition was observed to increase chlorophyll content. According to this situation the net photosynthetic rate increased. Both *in vitro* and *ex vitro* rooting can help a large scale micropropagation of oil palm.

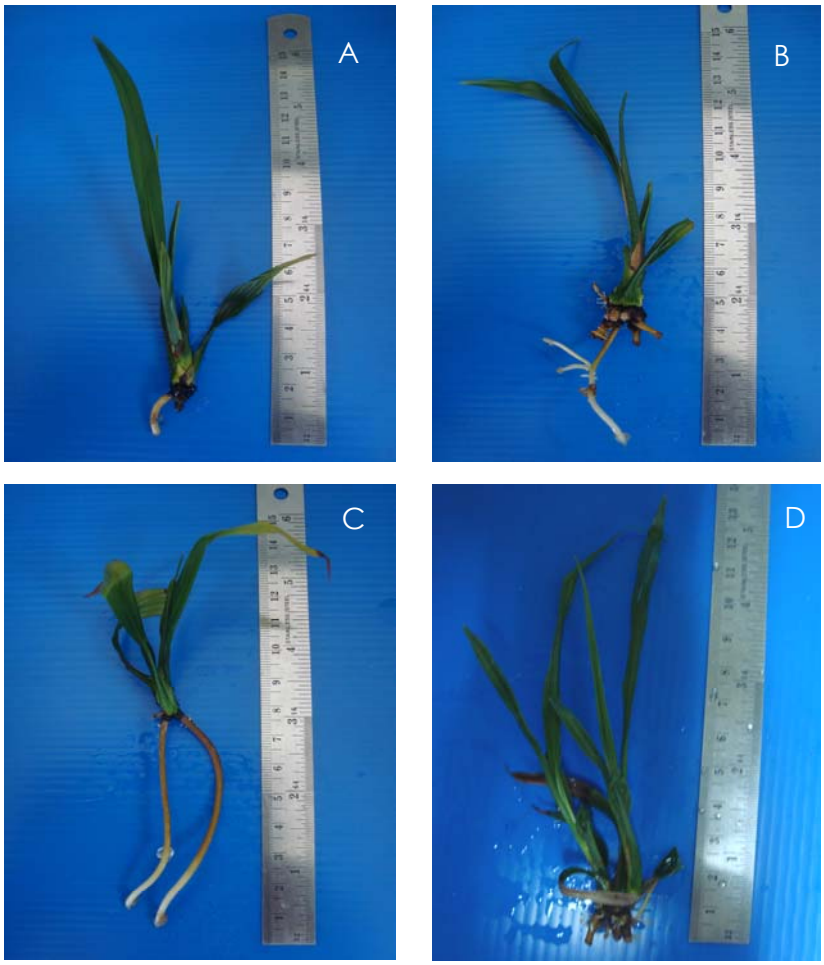

Figure 15 Root induced from *ex vitro* treatment after 12 weeks of culture.

A : control

B : Seradix#1 plus 3 mg/l PBZ

C : Seradix#1 plus 6 mg/l PBZ

D : Seradix# 1 alone.

3.3 Survival rate after acclimatization

Ex vitro rooting

After three months of *post vitro* growth, the percentage of plant survival differed among the treatments. From the five treatments of *ex vitro* root induction application of Seradix# 1 together with 6 mg/l PBZ gave the highest survival rate at 83-100% significant different to other treatments (Figure 16). Both Seradix# 1 and PBZ help the plantlets to resist the environmental stress after transplanting from axenic condition to normal cultivation in open pots. Only 43% of the non treated (control) plants could survive under this critical growth stage after three months of transfer.

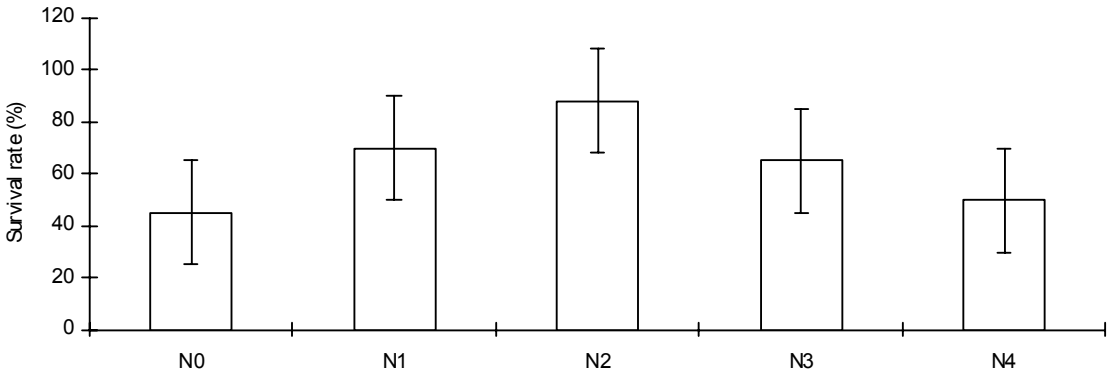

Figure 16 Survival percentage of *ex vitro* root induction after 3 months of transfer. (bar = SD)

N0: controlled treatment N1: Seradix#1 plus 3 mg/l PBZ

N2: Seradix# 1 plus 6 mg/l PBZ, N3: Seradx#1 plus 9 mg/l PBZ

N4: Seradix# 1 alone.

In vitro rooting

All the vitro-plants succeeded survived transplantation to the greenhouse. Additional of PBZ in the rooting medium significantly influenced. The micro-shoots rooted on the media containing PBZ were stronger with visibly thicker stem, which led to higher growth and establishment after 3 months of transfer in the greenhouse. The control shoot rooted without PBZ could not produce roots leading to

the failure of survival after transfer to *ex vitro* conditions. The vitro-plants were less stressed by changing of condition than treated plants. However, both *in* vtro and *exvitro* rooting plantlets could adapt well under greenhouse conditions (Figure 17).

Figure 17 Acclimatization of *ex vitro* rooting and complete plantlets of *in vitro* rooting under greenhouse conditions. Developmental stage treatment

A: control humidity by covering with glass bottle during 2-4 weeks

B: hardened plants can survive under greenhouse conditions after 30 days of acclimatization.

3.4 Root and leaf anatomy study

3.4.1 Leaf anatomy of vitro-plant of oil palm

The first indication of vigor of vitro-plant affected by PBZ is the presence of cell changing in anatomy of leaves. Leaves that occur on young plant are called juvenile leaves, whereas older ones are referred to as adult leaves. This difference can relate not only leaf size and shape but also to internal structure. In fact, it has been pointed out that in some instances, the leaves of a young plant may differ so much from those of the mature tree that they might appear to belong to different species.

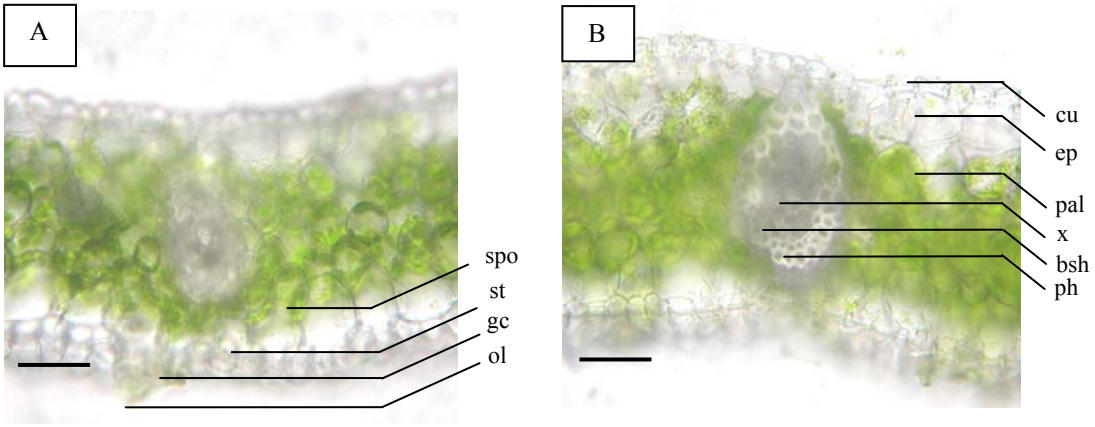

Figure 18 Transverse section of juvenile leaf from vitro-shoot of oil palm. (bar=100 µm)

A: control treatment B: treated with 6 mg/l NAA and 9mg/l PBZ

bsh: vascular bundle sheath; cu: cuticle; epi: epidermis;

gc: guard cell; ol: outer ledge; pal: palisade cell;

ph: phloem; spo: spongy parenchyma; st: stomata;

x: xylem.

From microscopic observation juvenile leaves developed from treated vitro-shoot had thicker epicuticular wax layer. The epidermal, palisade and spongy mesophyll cells were also larger. In addition, PBZ increased the width of the cortex and favored the formation of more secondary xylem vessel, resulting in thicker leaves (Figure 18). It was clearly shown that PBZ affected the change of bundle sheath cells of treated plantlets. Those cells were much larger than normal leaves. By the change of this cell structure it will facilitate the rate of water uptake of plantlets

3.4.2 Root anatomy of vitro-plant of oil palm

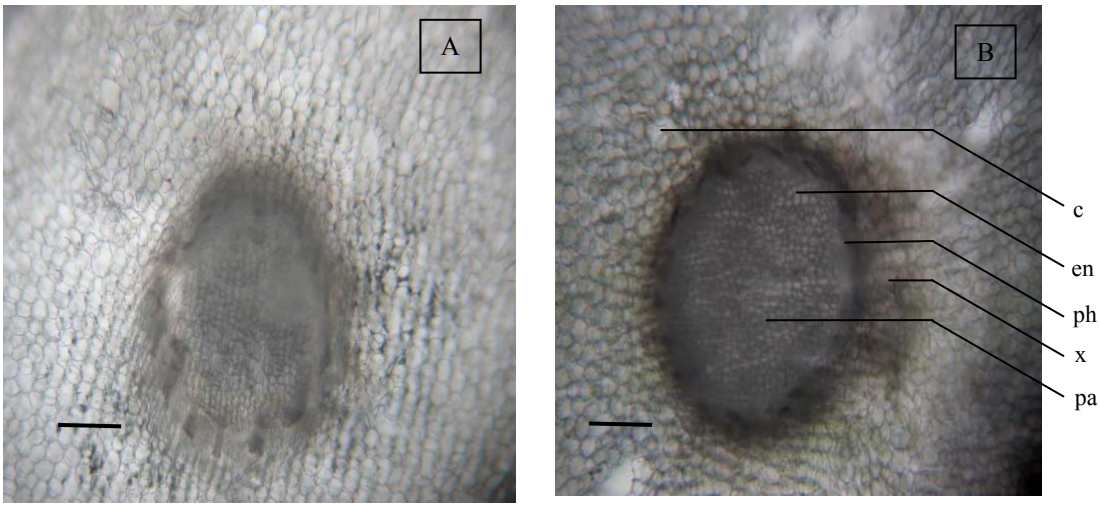

Figure 19 Transverse section of root from vitro-plant of oil palm. (bar=100 µm)

A: control treatment B: treated with 6 mg/l NAA and 9mg/l PBZ

c: cortex; en: endodermis; ph: phloem;

x: xylem; pa: stellar parenchyma.

Anatomical structure of root was similar to leaf anatomy. Well develop cell layers in root were obtained in root derived from culturing vitro-shoots on WPM supplemented with 7.2 % sucrose, 6 mg NAA and 9 mg PBZ. Microscopic observation showed significant thickness in cell size and increment of cortical cells or layer (Figure 19 B).

Chapter 4
Discussion

4.1 Effect of NAA and PBZ on root induction *in vitro*

4.1.1 Multiple shoot formation

The most important factors influencing *in vitro* shoot regeneration are plant growth hormones and the nutrient component of culture medium. However, in some species growth retardant can reduce size of shoot more than proliferating. Contrary result was obtained in this present study. PBZ, one of growth retardant, can induce a new forming shoot of oil palm in WPM in the presence of high concentration of sucrose (7.2%). Normally, shoot proliferating of oil palm was reported from EDS on liquid MS medium with decreasing the component to half of original concentration supplemented with low concentration of NAA and BA (Te-chato and Muangkaewngam, 1992). So far, there has no report on multiple shoot formation from single shoot culture of oil palm. It is amazing evidence that PBZ can promote the development of new shoot. PBZ may involve in endogenous hormone, especially cytokinin and giberellin which monitored new shoot bud formation.

The concentration of endogenous PGRs in tissue is determined by the relative rates of synthesis and the rate of consumption. In plant tissue culture, small explants are transferred onto culture media to make the PGRs needed for a developmental or growth response. However, by supplementing the medium with exogenous PGRs, it is possible to stimulate the responses that are desired from plant tissue.

Culture medium should be optimized for the first period of culture. In this period, PGRs has to adjust with high a concentration for dedifferentiation of culture explant and gradually decrease for further differentiation. The initial high concentration of PGRs might stimulate root initiation. Auxin has been shown to regulate different aspects of plant growth and development by affecting numerous processes including cell division, cell elongation and differentiation (Kochhar *et al*., 2008). Auxin is thought to be the major regulatory factors in the expression of specific genes affecting adventitious

rooting (Smith and Fedorof, 1995; El Euch *et al*., 1998). These signals may be involved (Calenza *et al*., 1995; Ermel *et al*., 2000). These signals could be responsible for different root developmental phase in the transition from meristems formation to be the initiation of apex organization and thereafter, root growth.

Regarding shoot induction, this process is clearly related to different morphogenic factor in this context, the fundamental role of the auxin and kind of triazole, especially NAA for auxin and PBZ derived from triazole in adventitious shoot stimulation. PBZ is azole derivative which had been found to promote the shoot inducing capability. PBZ at low concentration of 3 mg/l gave the highest shoot number of 3.5 shoots/ cultured shoot in korarima [*Aframomum corrorima* (Braun) Jansen] (Tefera and Wannakrairoj, 2006) while a very low concentration at 0.05-0.075 mg/l gave the best result in Friederick's Dendrobium (Te-chato *et al.,* unpublished data). In this present study, it was found that high concentration of PBZ (9 mg/l) was effective on shoot induction. In case of herbaceous gloxinia, it could tolerate to very high concentration of PBZ at 500 mg/l (Te-cahto and Chudecha, 2006). The different response to concentration of PBZ might be species specific. In addition, physiological status of different sources of explants (excised single shoot or shoot with few nodes) lead to the difference in balancing of indigenous phytohormones. Some authors reported that PBZ enhanced cytokinin activities and induced adventitious shoot proliferation in Araceae (Werbrouck and Debergh, 1996). Moreover, synergistic effects of PBZ and cytokinins, especially BA or TDZ were also reported on shoot proliferation (Werbrouck and Debergh, 1996; Tefera and Wannakrairoj, 2006). Unfortunately, cytokinins were not systematically used in combination with PBZ in this experiment. So, their synergistic effect on proliferation of oil palm in this study can not be postulated.

Adventitious shoot regeneration from leaf explants has been successful for various *Prunus* species. A complete protocol, for *Prunus avium*, with a high percentage of regeneration was achieved by optimizing hormone combination, leaf position and wounding (Bhagwat and Lane, 2004). Research emphasizing hormone

combinations have allowed the successful regeneration conducted by Anjana *et al*. (2005) about 83% rooting efficiency achieved from microshoot of *Cornus florida*.

There were numbers of study have been conducted on shoot regeneration affected by paclobutrazol. Podwyszynska and Marasek (2003) revealed that flower stalk explants of tulip was succeeded on multiple shoot formation at 70-100% when they were cultured PBZ and TDZ containing medium. Werbrouck and Debergh (1997) showed that paclobutrazol boosted the shoot inducing effect of cytokinins, BAP, in *Spathipyllum* sp. multiplied *in vitro*. PBZ combined with cytokinins was reported to induce strong shoot proliferation (Ziv 1990; Podwyszynska *et al*., 1998).

Hackett and Anderson (1967) got either single shoots from carnation shoot apices or proliferation of tissue from which shoots were later regenerated. Walkey and Woolfitt (1968) reported a similar kind of direct or indirect shoot proliferation from *Nicotiana rustica* shoot tips. Vine and Jones (1969) were able to transfer large shoot tips of hop (*Humulus*) to culture, but shoots only rooted, and showed a high proliferation of callus without a new shoot formation. Haramaki (1971) described the rapid multiplication of *Gloxinia* by shoot culture and by 1972 several reports of successful micropropagation by this method had appeared (Adam, 1972; Haramaki and Murashige, 1972). It was highly recommended by Edwin *et al*. (2008) that factors which have influenced the choice of shoot culture for practical micropropagation have been explained ; the way in which the method can be applied to wide range of different plant species, using the same principles and basic methods; (a) the possibility of obtaining simultaneous virus control; (b) a general uniformity and 'trueness to type' of the regenerated plants; (c) the relatively high rates of propagation which is possible in many species. Auxin has multiple roles in tissue culture, according to their chemical structure, their concentration, and the plant tissue being affected.

4.1.2 Growth of shoot

The essential function of auxin is to stimulate cell elongation, cell division in cambium tissue. Additionally, a high concentration of an exogenous auxin can induce

somatic embryogenesis. In our recently study, oil palm shoot cultured in WPM medium in the presence of PBZ showed higher length of shoot. PBZ at concentration of 9 mg/l in combination with 6 mg/l NAA promoted the highest shoot length at 11.4 cm, significant difference to other concentrations. Generally, it is necessary to root *in vitro* before acclimatization of plantlet to soil in order to convert the shoot to a functional plant that can be transferred to a greenhouse. Rooted shoot generally grow faster in culture, shoot generated *in vitro* root easily after transfer from the regeneration medium, which usually contain a high level of auxin.

Many plant growth retardants generally reduce elongation of internodes of higher plants both *in vitro* and *ex vitro*. In case of stem length, all PBZ-treated plants gave shorter internode due to the inhibitory effect on gibberellic acid production. It is active as a growth retardant in broad spectrum of species (Dalziel and Lawence, 1984; Lever *et al.,* 1982), especially in *Chrysanthemum x morifolium* (Barrett, 1982; McDaniel, 1983; Menhenett, 1984). Contrary result was obtained in our observation, that stem developed simultaneous by following root growth development. It should be realize, that after treated with PBZ the changes various plant parts, especially larger diameter of stem might be due to the increment in width of bundle sheath or storage of starch in parenchymatous tissue. Similar result was also described by Aguirre and Blanco (1990). However, McDaniel *et al.* (1990) found that PBZ caused a weaker stem. In potato, PBZ greatly reduced the portioning of assimilate to the leaves, stems, roots and increased portioning to the tubers compared to the control (Hazarika, 2001),. Contrary found that in this point study, PBZ increased the portioning of assimilate to the leaves, stems and roots.

The development of leaves obtained from various treatments of PBZ and NAA was quite different. This positive effect on leaf number became significant difference 4 weeks after culture. At the end of the experimental period, 6 weeks after culture confirmed the significant effect of various concentrations of NAA and PBZ on plant growth. During this stage mostly, the interaction of two main factors showed no significant difference at the early time of *in vitro* cultured. All plantlets had nearly same

number of leaves. New forming leaves emerged after 2 weeks of culture, but significant differences was not observed. Most of shoots developed new forming leaf after 6 weeks of culture. Few authors reported the effect of PBZ on leaf number per plant. In case of chrysanthemum, a low concentration (0.5 mg/l) promoted leaf number whereas higher concentration decreased those number (Smith *et al*., 1990).

Physiologically, length of axillary shoots produced in shoot cultures varies considerably from one kind of plant to another (Edwin *et al*., 2008). Species which have an elongated shoot system *in vivo* will produce axillary shoots which can easily separated as micro-cuttings and rooted individually. Root development was synchronized with shoot elongation and new leaf formation.

In several plant species, rooting remains one of the most critical stages in the micropropagation technique. However, in some species at least, shoots can reduced in size to little more than proliferating shoot initials by adding plant growth retardant (Ziv *et al.,* 1994) which are then suitable for large scale of culture consisting of superficial shoot meristems on a basal callus can sometimes be initiated from the base of shoots cultures.

Adding of NAA and PBZ for optimize developmental stage of vitro-shoot oil palm is the main objectives. The current study yielded that leaves obtained from treated shoot were thicker than those of control. The increased thickness might be due to increase in palisade and spongy mesophyll thickness like the report of Burrow *et al.* (1992). Moreover, PBZ increased epicuticular wax which closely related to leaf thickness. Burrows *et al.* (1992) treated *chrysanthemum* plants with PBZ as a soil drench resulted in thicker leaves, reduced stem diameter, and root with increase diameter. Sopher *et al* (1999) observed thicker and broader maize leaves having more epicuticular wax, enlarged vascular elements, and enlarged epidermal, mesophyll and bundle sheath cells due to PBZ treatment. Tonkinaon *et al*. (1995) has been noted that the application of PBZ reduce the length of wheat leaves by reduced cell length rather than cell number. Contrary result was obtained in this study which leaves grew more and fully supported to leaf development. Fortunately, there were no abnormal leaves

produced following treatment with PBZ. The increment in leaves width could be a result of leaf adaptation to low light intensity due to reduced shoot internode length and subsequent increasing canopy density, in order to allow more efficient capture of less available light (Proietti *et al*., 1997).

The choices some potent growth regulator presented in rooting media was reasonable. Morphologically, in attempt to produce great growth, moreover to passed successfully of critical condition because of root vitro plant malfunctioned were developed showed a weak. An interestingly result respect in this study, which was different from other report (Tekalign and Hammes, 2004), was internode length not affected by PBZ. In reality study, treated shoot with PBZ grew better than untreated by PBZ. This might be related to PBZ direct or indirect effect on cell morphology in the meristems region (Balsuka *et al*., 1993). Occasionally, the restriction on leaf and stem growth was not sensitive to the elevated of PBZ supplements in the range of 3 – 12 mg/l although they were effective on growth of control compared to PBZ free medium. However, developed root system shown by significant increase in root number with the elevation of PBZ concentration might be associated with the PBZ promoting photosynthesis relocation to the root system (Wang *et al* ., 1990).

4.1.3 Root formation

One problem encountered during *in vitro* condition especially for oil palm micropropagation are higher risk of rooting formation. Recently data shown that nearly than 40% roots were failure. However, in this study roots problems were solved by using some potent of plant growth regulator. The superior formula is WPM with 7.2% sucrose in cooperated with NAA and PBZ gave the greatest result in root induction from vitro-shoot at 93%. In addition, aberrant profile was observed *in vitro* shoots of oil palm affected by some potent regulators. These morphological changes were found in some abnormality occurred during *in vitro* growth impacted by higher of PBZ concentration.

From our previous studies root induction from excised single shoot in MS medium with 3 mg/l NAA and 1 mg/l 2i-P was never exceed 40%. By modification culture medium with low concentration of NAA and BA, root induction percentage

increased to nearly 75% (Te-chato and Muangkaewngam, 1992; Te-chato, 1998) Thus, the present study indicated that NAA and PBZ at optimum concentration in WPM medium with high concentration of sucrose was able to induce root better than MS medium with low concentration of sucrose. Geneve (1990) reported that PBZ can be enhanced adventitious root formation in English ivy and increase rooting ability of mung bean cutting. Sharma and Aier (1989) reported that exogenous application of auxin has a significant positive effect on adventitious root formation and 1,000 mg/l IBA significantly stimulated rooting of neem cutting (Palanisamy *et al*., 1998). Different plant species might vary in their requirement of auxin type for adventitious root formation. It was strongly indicated that NAA and PBZ in WPM medium were able to induce root formation of vitro shoot oil palm. However, *Santalum album* did not show any rooting response when inoculated on full-strength MS medium containing NAA (Kamil and Umboh,1992).

Besides of plant regulators treatment, sucrose level in medium were plays an important role in growth and development of root formation (Fuentes *et al.,* 2005). Sucrose is almost universally used for micropropagation purposes as it is so generally utilizable by tissue cultures. Refined white domestic sugar is sufficiently inhibits chlorophyll formation and photosynthesis making autotrophic growth less feasible. Selection of sucrose as the most suitable energy source for cultures follows many comparisons between possible alternatives. Sucrose has almost invariably been found to support growth equally well, and in a few plants it may result in better *in vitro* growth than sucrose, or promote organogenesis where sucrose will not, but will only be preferred for micropropagation where it produces clearly advantageous results (Edwin and George, 2008).

The superiority of plant growth regulator substances (PGRs) is essential to produce complete plantlets from micropropagation process, especially root induction. The efficiency of root induction depends on type and concentration of PGRs. The general superiority of sucrose over glucose for the culture of organized plant tissue such

as isolated roots may be on account of the more effective translocation of sucrose to apical meristems (Butcher and Street, 1964). In addition, there could be an osmotic effect, because, from an equal weight of compound, a solution of glucose has almost twice the molarity of a sucrose solution, and will thus, in the absence of inversion of the disaccharide, induce a more negative water potential.

The present study showed that application of auxin significantly improved rooting trait. The same result has been reported on many plant capable of rooting (Sharma and Aieir, 1989; Zeng *et al*., 2005). However, the promoting effect varied with auxin concentrations and types of auxin. Similar report was confirmed by Sadat and Henerty (2001) that maximum rooting of *Juglans regia* L (37.5%) was obtained on medium containing 4.0 mg/l IBA, he was confirmed that treatment containing IBA were better for rooting than those containing NAA.

The ability to produce roots is important for successful micropropagation it is positive result in healthy growth of root formation gave the high rate of survival after transferring to field condition.

4.1.4 Roots growth

Marino (1986) revealed positive effect of PBZ to the roots length S749 *Prunus persica* x S1490 *Prunus causuensis* during the first 14 days. After this period, PBZ had a negative effect on roots length according to its concentration. To support growth age, growth regulator and nutritional fluctuations are necessary to stimulate the proper developmental sequences of tissue cultured plantlets from bud initiation through stem elongation, and finally rooting (Mott and Amerson 1981).

Auxin control root elongation. It has been more difficult to demonstrate, perhaps because auxin induces the production of ethylene, a root inhibitor. However in this study by adding optimal concentration 2 – 6 mg/l NAA could promote the growth of intact roots, where inhibition effect observed at higher concentration up to 8 mg/l. Thus, roots may require a minimum concentration as described above especially for oil palm.

Hoessien *et al*. (2008) has been confirmed that the presence of auxin in rooting medium is sometimes needed for root initiation. NAA presented in the medium

was influenced significantly on root length. The use of plant growth regulators to improve crop productivity has interested plant scientist for many years. Moreover, the recent development of highly active growth retardants has further enhanced the potential uses of chemical growth regulators. Among those, PBZ is widely used. PBZ is classified as a member of triazole plant growth regulator group. It is believed that hormones play a vital role in the communication of signal between plant organs. All classes of plant hormones have some effect on one more aspects of the different steps leading for root formation. Although elongation of the primary root is inhibited by auxin at concentration higher than 10^{-8}M., initiation of lateral roots and adventitious roots is stimulated by high auxin levels. Lateral roots are commonly found above the elongation and root hair zone and originate from small group of cells in the pericycle. Auxin stimulates these pericycle cells to divide. The dividing cells gradually from into a root apex, and the lateral root grows through the root cortex and epidermis. Adventitious roots can arise in a variety of tissue locations from clusters of mature cells that renew their cell develop into a root apical meristems in a manner somewhat analogous to the formation of lateral roots. There is several point of evidence indicating high assimilate level is a contributing factor in induction besides hormonal factors. Khan *et al.,* (2006) revealed that root induction of sugar cane could be performed by adding 6% sucrose to the growing medium. In root formation, sucrose was found superior than other carbon sources (Hossain *et al*., 2005). High sucrose level increases the osmotic potential of the media and enhances starch accumulation (Nawsheen, 2001).

Root formation is also influenced by genotype and environmental interaction (Davis *et al.,* 1988). Growth regulator and nutritional fluctuation are necessary to stimulate the proper developmental sequences of tissue cultured plantlets from vitro shoot and finally rooting (Mott and Amerson 1981). Environment factors such as light quality, photoperiod, CO_2 concentration, O_2 concentration, pH, osmotic potential, relative humidity, temperature, plant growth regulator, and nutrients can have dramatic effects on rooting process and relatively well understood, at least in terms of modifying root production (Haissig *et al*., 1992).

Root will be produced if plantlets fully adequate nutrient and several supporting factors. In order to obtained quality plantlets with less mortality rate, more root formed from each plantlet is needed. In priority case of vitro-shoot of oil palm as EDS, totally half vitro-shoot, poor rooted. Physiologically, root as tool for uptake nutrient and water is a main factor for growth sustainable passed on critical condition. Potent growth regulator was chosen in our experimental study NAA and PBZ could be able to altered more number of root. The interesting, this condition continuously occurred in *ex vitro* condition for empowering survival rate in critical condition.

The synergic ability of plant growth could be able to support plants which were malfunctioned in previous studies because of a good shoot and root system becoming future with completed plantlets. Hopefully, this complete protocol capable to promote a millions of healthy normal plantlets with lower labors cost. Additionally, the advantages of this protocol could be applied for other species of palm.

4.1.5 Chlorophyll content

Chlorophyll content is one of the direct markers of the efficiency of the photosynthesis apparatus, informing as it does about all chlorophyll and not only that which takes part in photosynthesis. Photosynthesis is a physiological marker related to vitro-plant quality (Borkoska, 2003). In this current study they were statistically significant between the control and treated-shoots with 6 mg/l of NAA and 9 mg/l of PBZ. This is real evident that PBZ treated vitro-plants are better prepared for photosynthesis and this usually occurs during their *in vitro* development and continuously to the greenhouse.

Combination of NAA and PBZ treatment increased chlorophyll content compared to control. PBZ treated shoots gave chlorophyll nearly two times higher than control. Similar results were also reported in barley seedling (Sakar *et al*., 2004) and tomato (Still and Pill, 2004). New forming from PBZ treated shoot were dark green due to high chlorophyll a, b and total chlorophyll (Sopher *et al.,* 1999). Fletcher *et al*. (2000) proposed that PBZ as one in triazol group stimulate cytokinins synthesis that enhances chloroplast differention, chlorophyll biosynthesis and prevents chlorophyll degradation.

Moreover, Te-chato *et al*. (2008) proved that KN was necessary for generation of chlorophyll in oil palm cell suspension culture. The mechanism of KN or cytokinins on chloroplast formation was not clearly understood. It might involve in cell division and some protein synthesis in relation with chloroplast development. In this present study, it is suggest that PBZ may involve in the formation of cytokinins, especially kinetin.

PBZ applied on embryo derived shoot were consistently supported in chlorophyll *a* and *b* contents. Study on chlorophyll content was conducted by Sebastian *et al.* (2002). They reported that PBZ enhanced chlorophyll synthesis in *Dianthus caryopphyllus*. Khalil (1995) observed more densely packed chloroplasts per unit leaf area in response to PBZ treatment. Increased chlorophyll content in potato due to PBZ treatment was observed by Balamani & Poovaiah (1985) and Bandara and Tanino (1995). The higher chlorophyll content of treated-potato leaves may be related to the influence of PBZ on endogenous cytokinins levels. It has been proposed that PBZ stimulates cytokinins synthesis that enhanced chloroplast differentiation, chlorophyll biosynthesis, and prevents chlorophyll degradation (Fletcher *et al*., 1982).

Effect of sucrose concentration on photosynthetic activity of *in vitro* culture study was conducted by Jan *et al.* (2007) who reported that the 0.3% sucrose was the most effective for functionality of concentration of sucrose was altered expected result for root induction. Concentration of sucrose normally used to support the growth of tissue cultures are often inhibitory to chlorophyll synthesis (Rier and Chen, 1964; Edelman and Hanson, 1972) but the species of plant from which the tissue was derived. Shininger (1979) has concluded that only carbohydrates which capable of promoting treachery element formation. Sugars apart from sucrose are not inhibitory (Edelman and Hanson, 1972; El Hinnawiy, 1974). Similar results have been obtained for various species (Kovtun and Daie, 1995), and they could simply reflect that the additional carbohydrate pool was used for biomass formation. Also the accumulation of chlorophyll and the photosynthetic capacity were positively affected by sugar feeding.

An increase in photosynthesis occurs when plantlets are growing (Short *et al*., 1987), but these treatment are successful in ensuring greater survival of plantlets when they are transferred *ex vitro* condition.

4.2 Effect of some potent PGRs on root induction *ex vitro*

The successful acclimatization of micropropagated plants and their subsequent transfer to the field are crucial step for commercial exploitation of *in vitro* technology (Pati, 2006). It was quite different that a high survival rate in transplanting shoot vitro oil palm plantlets mainly due to the marked difference in relative humidity between *in vitro* and *ex vitro* conditions. So acclimatization of rooted shoots was necessary before they were taken out of the flask. In this current study, all rooted plantlets can be transplanted in the greenhouse, the survival rate of completed plantlets developed up to 93%. This significant different could be due to the fact that *ex vitro* roots are more functional as a consequence of their anatomical characteristics. These characteristics may be advantageous both for water absorption and translocation. It is know that plant growth results in the production of more durable, compact plants with stronger shoots and roots.

The similar study revealed by Hazarika *et al*. (1991) that preconditioning citrus microshoots with PBZ influence higher *ex vitro* survival by intensifying internode length, thickening of root and reducing leaf dehydration by regulating the stomatal function and increasing epicuticular wax per unit area of leaf, besides more chlorophyll synthesis.

Acclimatization is a crucial and difficult stage in micropropagation in vitro shoot oil palm, specifically to get root with good characteristic. It should be concerned that with a quality of root produced plays an important role for successful transfer of plantlet to the soil during acclimatization. The occurrence of incomplete vascular connections between the shoot vitro and the *in vitro* developed roots, which restricted water movement and consequent growth, has been reported (Grout and Aston, 1977; McClelland *et al*., 1990). According to these authors, this fact could be an important cause of the higher mortality percentages during the acclimatization stage, this

physiological disorder being more pronounced if root not well developed and additionally vascular connection are malfunctioned. In this review study vitro-shoot rooted on rooting media capable to relocate in the field condition, however those plantlets not fully formed the root yet but the plant gradually well adapted in *ex vitro* conditions. In order to make suitable condition, the presence of lateral roots and root hairs developed in natural substrate may have a positive influence. As a result of all these factors, the microplants with the root system developed in peat: perlite substrate respond fairly satisfactorily when they are placed in acclimatization condition.

In other to keep plantlet in well condition Hazarika *et al*. (2000a 2001a) also reported that preconditioning microshoots with PBZ influence higher *ex vitro* survival by intensifying internode length, thickening roots and reducing leaf dehydration by regulating stomatal function and increasing epicuticular wax per unit area of leaf of *in vitro* cultured citrus plantlets. PBZ is among the compounds that can positively affect the acclimatization of microplants (Smith *et al*., 1990; Robert *et al*., 1992; Gilley and Flecther, 1997; Sopher *et al*., 1999). The following effects were found as results of application of PBZ *in vitro* increases in the chlorophyll content, the net photosynthetic rate, the internal CO_2 concentration, the thickness of the leaves, cuticle, palisade and spongy parenchyma, and the diameter of the phloem elements, a decrease in the diameter of the xylem vessels, reduction in transpiration rate (Jaleel *et al*., 2007) and shoot length, and an improvement of rooting (Messina and Costa, 1990).

The most concerned for acclimatization is environmental factors such light quality, photoperiod, concentration, O_2 concentration, pH, osmotic potential, relative humidity, temperature, plant growth regulators, and nutrients can have dramatic effects on rooting processes and are relatively well understood, at least in term of modifying root production (Haissig *et al*., 1992). Because root formation also influenced by genotype and environmental interaction (Davis *et al*., 1988). In contrast to environmental influences, genotypic variability in rooting potential is less well understood.

Acclimatization of micropropagated plants to a greenhouse or a field environment in essential because there is difference in micropropagation environment and the greenhouse of field environment. Successful acclimatization procedures provide optimal conditions for higher survival, subsequent growth and establishment of micropropagated plants. The physiological and anatomical characteristics of micropropagated plantlets necessitate that they be gradually acclimatized to the environment (field conditions). Techniques that are more satisfactorily address the changes required for successful acclimatization require lower relative humidity, higher light level, autotrophic growth and a septic environment that are characteristic of the green house. Although specific details of acclimatization may differ, certain generalizations.

4.3 Leaf and root anatomical study

The first indication of vigor vitro-plant affected by PBZ is the presence of cell changing in anatomy of leaves. New forming leaves developed from vitro-shoots were thick and wide so called juvenile leaves, whereas older leaves were referred to as adult leaves. This difference related not only leaf size but also the internal structure. In fact, leaves from untreated plants were a darker green and were thicker than those of control. Similar result was also reported in treatment with PBZ (Burrow *et al.,* 1992). However, increased thickness in *Chrysanthemum* leaves was due to increases in palisade (64%) and spongy mesophyll (72%) whereas increased thickness in oil palm leaves in this present study was due to increases in cuticle layer and bundle sheath. Individual bundle sheath cells were larger or bigger in the PBZ-treated shoots together with two to three layers of cuticle resulted in thicker leaves. Studying leaf anatomy conducted by Ahmad Nazarudin *et al*. (2006) from analysis of variance and Turkey's studentized range test showed that there was significant increase (p.0.05) in palisade parenchyma thickness of leaves of plants treated with 3.75 g/l PBZ compared to leaves of non-treated plant. On the other hand, the spongy parenchyma thickness of *S. campanulatum* was not affected by the application of this triazole compound.

According to Gao *et al.* (1987) triazole have several morphological effects on leaves thickness and epicuticular wax. Tekalign (2005) reported that PBZ increased length and width of palisade mesophyll cell of tomato leaves. Burrows *et al.* (1992) reported that increased *Chrysanthemum* leaf thickness in response to PBZ treatment was due to induction of additional layers of palisade parenchyma, although individual cells were shorter, of small diameter and more tightly packed.

Control plants possessed tap root system, while PBZ treated plants and had fibrous root system. Low concentration of PBZ (3-6 mg/l) gave a medium size of fibrous roots whereas high concentration (12 mg/l) had many large diameter roots. The medium fibrous thickening of the roots obtained from PBZ-treated plants is common phenomena (Barnes *et al*., 1989; Bausher and Yolenosky, 1987). The present study supports to these observation and suggests that whether or not the metabolism of endogenous cytokinins is influenced, especially, cell division and enlargement. The number of roots and their thickness were greatly increased at particular high concentration of PBZ. PBZ increased root diameter by increasing the width of cortex and by favoring the formation of more secondary xylem vessels. Depending on the plant species and PBZ concentration, PBZ either stimulated or inhibited root growth. A simple, economical and efficient method of application capable of yielding consistent results is the top priority in the utilization of plant growth regulators for commercial purpose.

There was progression in this study, while root stimulated by PBZ and NAA this occurred might be the meristemoids enlarged in volume and cell number increased as a result of their division. The appearance of this meristemoids signaled the initiation of adventitious roots. In case of apple (Naija *et al*., 2008) the processes of initiation and development of adventitious root were not synchronous. Different stages of adventitious root developing in single pieces of stem were observed at the same time, and not all meristemoids developed into adventitious roots. Root primordial had developed a vascular system, which was continuous with that of the stem. Moreover, cells leading to root formation could have phloem parenchyma. The region of the tissue in which cells becomes activated is though to depend in part on physiological gradients

of substances entering the shoot from the medium, and on the presence of competent cells to respond.

Chapter 5
Conclusion

5.1 Effect of some potent PGRs on shoot formation and root induction

EDS oil palm cultured on WPM medium supplemented with 7.2% sucrose, 6 mg/l of NAA and 9 mg/l PBZ gave the highest result in both number of shoot and shoot growth. The number of shoots obtained in those PGR containing WPM was ranging from 2-3 shoot/ cultured shoot after 6 week of cultured.

The highest shoot length (11.4 cm), stem width (0.5 cm), leaf number (5.7 leaves/shoot) and leaf width (1.2 cm) was obtained from WPM media supplemented with 6 mg/l NAA and 9 mg/l PBZ after 12 weeks of culture.

For *in vitro root induction* the highest root formation in term of percentage (93), root number (6-7 roots/cultured shoot) and root length (3-4 cm) was also obtained from WPM media supplemented with 6 mg/l NAA and 9 mg/l PBZ after 12 weeks of culture. The characteristic of root was fibrous root system.

5.2 Acclimatization

For *ex vitro* root induction Seradix#1 with 6 mg/l PBZ gave the best result but far lower than *in vitro* treatment.

Survival of the plantlets after transfer to soil was more than 90% for *in vitro* rooting and 80% or less for *ex vitro rooting*.

PBZ at concentration of 9 mg/l with 6 mg/l NAA gave the highest chlorophyll a (2.32 mg/gFW), chlorophyll b (1.76 mg/gFW) and total chlorophyll (3.54 mg/gFW).

5.3 Leaf and root anatomy

PBZ-treated EDS promoted thicker epicuticular wax layer. The epidermal, palisade and spongy mesophyll cells were larger. In addition PBZ increased the width of the cortex and favored the formation of more secondary xylem vessel, resulting in thicker roots.

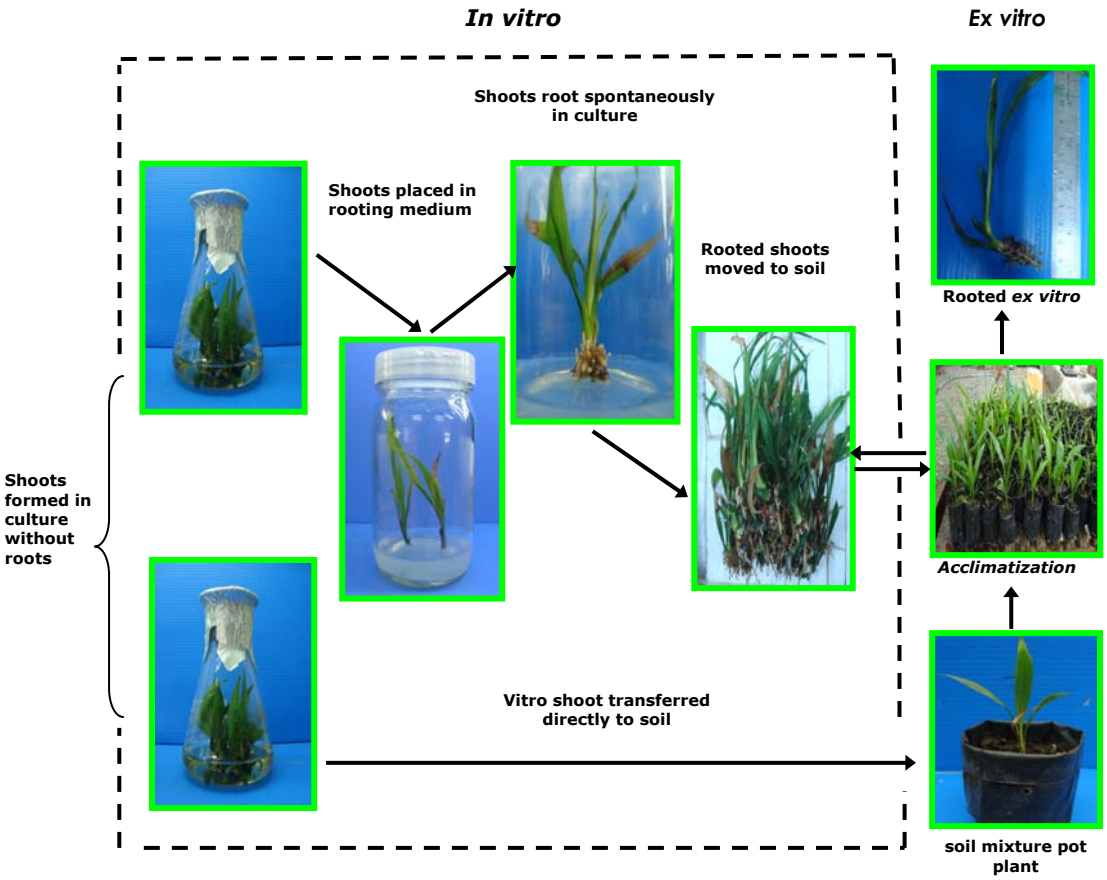

Figure 20 Methods for rooting *in vitro* and *ex vitro* of micropropagated vitro-shoots of oil palm.

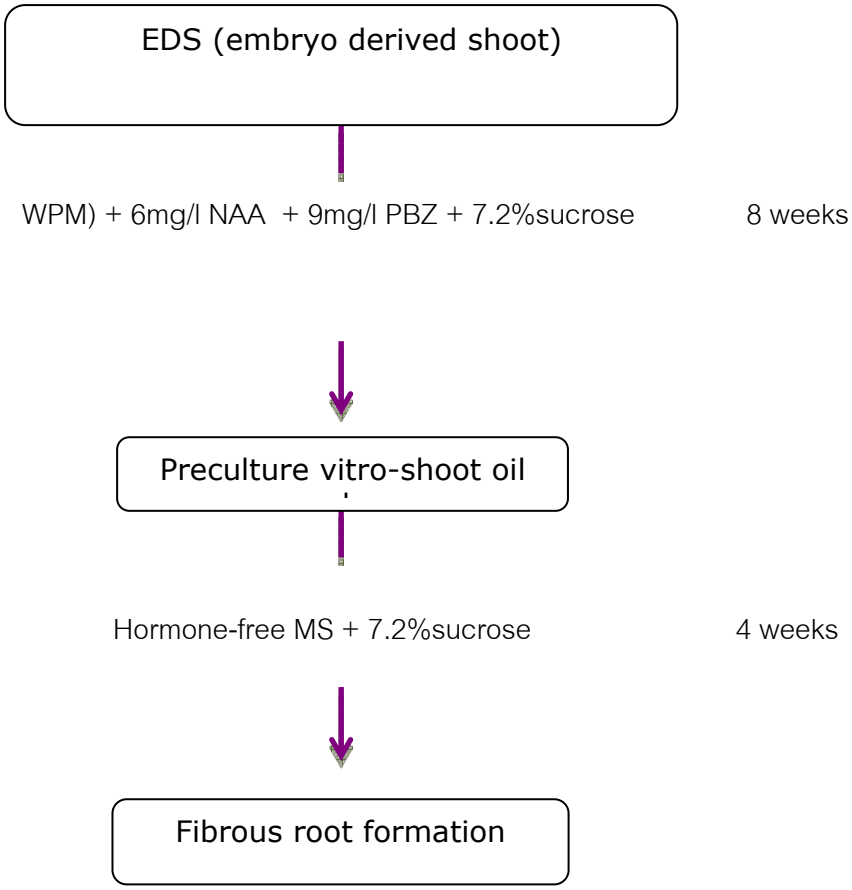

Figure 21 Diagram of efficient protocol for root induction from vitro-shoot of oil palm.

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