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The effects of different concentrations of NAA on oil palm (*Elaeis guineensis*) embryoid cultures and phytosterols production

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Abstract

Oil palm tissue culture is subjected to indirect embryogenesis which involves five stages including mother palm selection and preparation, establishing an aseptic culture, multiplication of suitable propagules, formation of shoots and roots, and transfer to natural environment. This study aimed to investigate the effects of NAA concentrations on further improvement of oil palm (*Elaeis guineensis*) embryoid cultures of three selected Malaysian Palm Oil Board (MPOB) clones (PL 213, PL 209 and PL 220) and phytosterols production. Different concentrations (0, 0.5, 1.0, 2.0 mg L^{-1}) of NAA were added to the MS basal medium and the cultures were incubated at 27 ± 2°C and 12 hours light/day photoperiod. Measurements on fresh weight of embryoids and number of shoots were recorded every two weeks throughout 16 weeks of experiment. The results indicated that PL 213 gave the highest mean of fresh weight (55.3±3.4 g) and number of shoots (123±4) in MS medium without NAA (control) followed by clones PL 209 and PL 220. There is a significant difference of clone and NAA concentration on the fresh weight of embryoid cultures but there is no significant effect of NAA concentration on the number of shoots. However, high concentration of NAA up to 2.0 mg L^{-1} had stimulated tissue necrosis and excretion of phenolic compounds into the culture medium. In the phytochemical study, clone PL 213 also produced the highest phytosterols in type and percentage. The useful sterol compounds that present in the extracts of PL 213 embryoid were γ-sitosterols, β-sitosterols, stigmasterol, campesterol, stigmasterol, 22,23-dihydro- and stigmasta-7, 22-dien-3-ol, (3.beta.,5.alpha.,22Z)-. It has been observed that higher concentration (1.0-2.0 mg L) of NAA gave poorer effects on the growth and the phytosterols production in all oil palm clones compared to control and 0.5 mg L^{-1} NAA used in this study.

Keywords: Embryoids; Genotype; NAA; Oil palm; Phytosterols; Shoots.
Abbreviations: NAA_naphthaleneacetic acid; MS_Murashige and Skoog.

Introduction

Oil palm (*Elaeis guineensis*) is a crop species producing high quality oil, which can be found from the fruit mesocarp (palm oil) and the kernel of the nut (palm kernel oil). It takes second place only after soy bean in the world's supply (Kantamaht et al., 2010). Propagation *in vitro* has great advantages in the case of the oil palm which is vegetative propagation is possible only via tissue culture. The plant cannot be multiplied vegetatively since it is a monocotyledonous species with a single growing apex (Rajesh et al., 2003). Oil palm (*Elaeis guineensis* Jacq.) planting materials had, until recently, been produced by sexual means through *dura* x *pisifera* (DxP), resulting in the *tenera* offspring (Zamzuri, 2011). The switch from planting *dura* to *tenera* (or DxP) about half a century ago resulted in a quantum leap of 30% in oil yield through manipulation of a single gene. Therefore, cloning of the oil palm seems to be the best approach to capture the maximum potential of a selected genotype in order to produce a large number of high quality oil palm ortets. Cloning is a process in which identical or true-to-type 'photocopies' of a selected palm (ortet) are reproduced by developing plantlets from the leaf tissue of *tenera* oil palms with desirable characteristics. Clonal oil palm offers the potential for greater productivity because it is possible to establish uniform tree stands comprising identical copies (clones) of a limited number of highly productive oil palms (Mutert and Fairhurst, 1999). Khaw and Ng (1997) reported that the performance clonal plantlets derived from selected ortets were significantly superior to the commercial DxP seedlings and the clones yielded at least 20% more than seedling standards (Kushairi et al., 2006). According to De-Touchet et al. (1991), micropropagation of oil palm is the promising approach with improved agricultural traits where clonal multiplication of elite plants can be produced. MPOB has developed the basic protocol for liquid culture (Tarmizi, 2002) which includes selection of a suitable callus (friable type), media formulation, aggregate sieving, maturation induction, embryoid regeneration and production of rooted plantlets in a dual phase system (solid and liquid media). The development of somatic embryo into normal plantlet commonly occurs through four stages *i.e.* callus induction, callus proliferation, embryo maturation and germination (Otih and Sitti, 2008). The somatic origin of the embryoids was obtained from meristematic cells which were highly differentiated with numerous storage lipids (Kanchanapoom and Tinnongjig, 2001). Most of somatic embryos were yellow-greenish in color with smooth surface and more compact (Sumaryono et al., 2008). Embryo germination was indicated by the presence of shoot and root buds (Otih and Sitti, 2008) while Goh et al. (1999) stated that embryo germinated when the

plumullae started to emerge. Cleavage polyembryony is the pattern of embryogenesis where embryos arising from such groups of cells originated from a single embryogenically-determined cell (Haccius, 1978). Appropriate composition and concentration of growth regulators are able to improve somatic embryo development (Gaspar et al., 1996; Tahardi et al., 2003). Commonly used synthetic auxins in tissue culture are 2,4-dichlorophenoxyacetic acid (2,4-D; often used for callus induction and suspension cultures), and 1-naphthaleneacetic acid (NAA; when organogenesis is required) (Thomas et al., 1996). Various types of auxins – IAA, 2,4-D, NAA and 2,4,5-T were used to develop a tissue culture protocol particularly for oil palm (Wooi, 1995). The general protocol uses NAA as exogenous auxin in the early culturing stages (Rohani et al., 2003). The switch from 2,4-D to NAA was encouraged as cultures on 2,4-D were particularly prone to genetic variation (Machakova et al., 2008; Sogeke, 1998; Sogeke et al., 1999). This strongly indicates that NAA seems to be appropriate for growth improvement in oil palm tissue culture. Phytochemicals are the natural bioactive compounds found in plants and these phytochemicals are basically divided into two groups, i.e. primary and secondary metabolites; according to their functions in plant metabolism (Dipak et al., 2010). Secondary metabolites are chemicals produced by means of secondary reactions resulting from primary carbohydrates, amino acids and lipids (Ting, 1982). Plant secondary metabolites have chemical and pharmaceutical properties interesting for human health (Raskin et al., 2002; Reddy et al., 2003). *In vitro* plant tissue cultures have long been viewed as a source of commercially important steroids, alkaloids and terpenes for pharmaceutical industry (Bohm, 1980; Staba, 1980; Barz and Eills, 1981; Deus and Zenk, 1982). The evolving commercial importance of the secondary metabolites has in recent years a great interest, in secondary metabolism, and particularly in the possibility to alter the production of bioactive plant metabolites by means of cell culture technology. The principle advantage of this technology is that it may provide continuous, reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant cells from which these metabolites can be extracted (El-Dawayati et al., 2012). Steroidal compounds are of importance and interest in pharmacy due their relationship with such compounds as sex hormones (Okwu, 2001). According to Delazar et al. (2010), phytosterols are known to possess properties such as being anti-fungal, insect-deterrent, mould-inhibiting and antimicrobial (Gus-Mayer et al., 1994; Morrisey and Osbourn, 1999). Piironen et al. (2000) reported that sterols also act as substrates for certain secondary metabolites such as glycoalkaloids, cardenolides and saponins. Oil palm is a folk remedy for cancer, headaches, and rheumatism, and is considered an aphrodisiac, a diuretic, and a liniment (Irvin, 1985). Sreenivasan et al. (2010) revealed the presence of tannins, alkaloids, steroids, saponins, terpenoids, and flavonoids in the methanolic oil palm leaf extract whereas the extract has strong wound healing capacity. Thus, this study aimed to investigate the effects of different concentrations of NAA on oil palm (*Elaeis guineensis*) embryoid cultures and phytosterols production.

Results and Discussion

Effect of different concentration of NAA on fresh weight and number of shoots

This study indicates that fresh weight and number of shoots for clones PL 213, PL 209 and PL 220 were increased when the cultures were maintained for a longer period of time as illustrated in Figure 1 and 2. From the observation, the fresh weight of embryoid cultures was increased vigorously from week 4 to week 16 (Figure 1) while the shoot formation started to appear at the second week of culture and developed continuously from week 4 to week 12 (Figure 2). However, the development of shoots had slow down after 12 weeks. The highest mean of fresh weight (55.3±3.4 g) and number of shoots (123±4) was achieved by clone PL 213 in MS media without NAA (control). The lowest mean of fresh weight (34.0±3.1 g) was achieved by clone PL 220 supplemented with 0.5 mg L^{-1} NAA while the lowest mean of number of shoots (36±4) was achieved by clone PL 220 supplemented with 2.0 mg L^{-1} NAA. There was an interaction of clone and NAA concentration on the fresh weight of embryoid cultures and the number of shoots after 16 weeks of culture. Besides, there was a significant difference on the fresh weight among three different clones; PL 213, PL 209 and PL 220. Also, there was an effect of NAA concentration on the fresh weight. There was also a difference on the number of shoots among three different clones; PL 213, PL 209 and PL 220 while there was no significant effect of NAA concentration on the number of shoots. Generally, these results showed that embryoids maturation and germination of three oil palm clones responded differently to various NAA concentrations used in this study. PL 213 gave the highest fresh weight and number of shoots without supplemented with NAA (Figure 3). However, the effect of NAA was not similar with the other two clones; PL 209 and PL 220. It showed that growth and development of the cultures might vary from clone to clone whereas Haliza Dahlia et al. (2011) also found that different clones of oil palm cell suspension have different rate of growth. Organogenesis and somatic embryogenesis are depending on genotype and hormonal application (El-Bellaj, 2000). This study found that high concentration of NAA (2.0 mg L^{-1} NAA) was not suitable for oil palm embryoids growth and development. Tisserat (1984) used medium supplemented with NAA and BA for shoot formation while Rodriguez and Wetzstein (1998) revealed that the development of somatic embryogenesis of *Carya illinoinensis* (Wagenh) C. Koch was induced by different auxins through morphological and anatomic analysis, in which auxins NAA and 2,4-D induce accentuated cell division in the subepidermal layer of the cotyledons of immature embryos. Kuberski et al. (1984) reported that the auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and NAA triggered somatic embryogenesis and improved the later development of embryos in *D. lanata*.

Embryoids development, necrosis and phenolic compounds

Embryoids maturation and germination in all of media treatments was observed in oil palm clones PL 213, PL 209 and PL 220 with high frequency (70-100%). All clones gave 100% of embryoids maturation, while PL 213 gave the highest frequency of embryoids germination (100%) followed by PL 209 (90-100%) and PL 220 (70-90%). Sumaryono et al. (2008) reported that oil palm somatic embryos at early developmental stage (globular and heart-shape) were observed to become a numbers of somatic embryos (polyembryoids) at advanced developmental stages (torpedo and cotyledonary) after 6 weeks of culture.

After four weeks, the color of embryoids of all three clones has started to turn into brown. However, the number of shoots has also increased as PL 213 culture showed the most vigorous growth compared to PL 209 and PL 220. Previous finding showed that embryoids maturation was indicated by

Table 1. Phytosterols detected in fractions of MeOH extracts of oil palm embryoids after 16 weeks of culture.

Clone	Treatment	Compound	Retention time (min)	Area, %	Molecular mass	Molecular formula
PL 213	MS0	Stigmasterol, 22,23-dihydro-	27.27	4.66	414	$C_{29}H_{50}O$
		γ-sitosterol	27.27	4.66	414	$C_{29}H_{50}O$
	MS1	Campesterol	26.34	1.14	400	$C_{28}H_{48}O$
		Stigmasterol	26.66	1.53	412	$C_{29}H_{48}O$
		Stigmasta-7, 22-dien-3-ol, (3.beta.,5.alpha.,22Z)-	26.66	1.53	412	$C_{29}H_{48}O$
		Stigmasterol, 22,23-dihydro-	27.31	3.32	414	$C_{29}H_{50}O$
		γ-sitosterol	27.31	3.32	414	$C_{29}H_{50}O$
		β-sitosterol	27.31	3.32	414	$C_{29}H_{50}O$
	MS2	Campesterol	26.33	1.32	400	$C_{28}H_{48}O$
		Stigmasterol, 22,23-dihydro-	27.29	10.76	414	$C_{29}H_{50}O$
		γ-sitosterol	27.29	10.76	414	$C_{29}H_{50}O$
		β-sitosterol	27.29	10.76	414	$C_{29}H_{50}O$
	MS3	Stigmasterol	26.58	0.39	412	$C_{29}H_{48}O$
PL 209	MS0	Stigmasterol	27.23	6.28	412	$C_{29}H_{48}O$
		β-sitosterol	27.23	6.28	414	$C_{29}H_{50}O$
	MS1	Stigmasterol, 22,23-dihydro-	27.26	2.78	414	$C_{29}H_{50}O$
	MS2	NP				
	MS3	NP				
PL 220	MS0	γ-sitosterol	27.22	5.03	414	$C_{29}H_{50}O$
		Stigmasterol	27.22	5.03	412	$C_{29}H_{48}O$
		β-sitosterol	27.22	5.03	414	$C_{29}H_{50}O$
	MS1	NP				
	MS2	γ-sitosterol	27.21	4.33	414	$C_{29}H_{50}O$
		β-sitosterol	27.21	4.33	414	$C_{29}H_{50}O$
	MS3	NP				

Note: NP = not present; MS0: MS media without NAA (control); MS1: MS media supplemented with 0.5 mg L^{-1} NAA; MS2: MS media supplemented with 1.0 mg L^{-1} NAA; MS3: MS media supplemented with 2.0 mg L^{-1} NAA.

the change of embryo color and formation of the brown spots. The brown spots suggest the presence of an active substance *i.e.* amylum, protein and lipid, secreted by somatic embryo from the osmotic cell pressure. The increase of secretion of active substances suggests its correlation with the desiccation process when somatic embryo enters the germination phase (Otih and Sitti, 2008). The culture medium of MS+2.0 mg L^{-1} NAA turned into brown color as the age culture was increased. Phenolization is a relatively frequent phenomenon that occurs during the *in vitro* establishment of some plant species whereas it is a result of the exudation of phenolic compounds that oxidize in contact with the air, forming a dark precipitation (Araceli et al., 2012). The accumulation of these toxic compounds can affect tissue viability and when severe can lead to necrosis (Pierik, 1998). Compton and Preece (1988) found that phenolic compounds exuded from excised explants were oxidized by peroxidases or polyphenolo-xidases, causing browning of both plant tissues and media. This might reduce growth or kill the tissues (Preece and Compton, 1991). Accumulation of polyphenolic compounds in embryogenic tissues during somatic embryogenesis has been previously reported in many plants (Kouakou et al., 2007; Reis et al., 2008). Various shapes of normal embryoids either a single normal or fused embryoids, had been differentiated into plantlets. However, some abnormal changes were identified on embryoid cultures of clones PL 209 and PL 220 supplemented with 2.0 mgL^{-1} of NAA. This type of embryoids probably underwent necrosis as it became soft, dark and finally aborted without any further development. Thus, 2.0 mgL^{-1} of NAA appeared to be too high for oil palm embryoid cultures because it can cause necrosis to the plant tissues and accumulation of phenolic compound. However, embryoids necrosis and production of phenolic compound might also be influenced by several factors such as clone, age and plant growth regulators. Previous studies reported that factors influencing necrosis were the growth temperature (Marc, 1990), plant growth regulator (Bairu et al., 2011; Madhulatha et al., 2004), age and cultivar (Martin et al., 2007). Other than that, Gow et al. (2009) reported negative effects on direct somatic embryogenesis of *Phalaenopsis* orchids in cultures exposed to light; in addition, light induced embryo necrosis and low plantlet regeneration. However, reduction of necrosis by more frequent subculturing has been demonstrated in *Prunus tenella* (Alderson et al., 1987). Also, pretreatment of explants with the antioxidants had a positive effect by reducing the degree of tissue necrosis and reducing the oxidation of phenolic compounds (Ravindra and Johannes, 2005).

Identification of phytosterols in oil palm embryoids using GC-MS

The results of the GC-MS analysis on the identification of sterol compounds were presented in Table 1. A total of 6 sterol compounds were identified from the crude embryoid extracts of oil palm and the retention time for each type of sterol compounds was found between 26 to 28 minutes. However, the presence of each sterol compounds was different among clones PL 213, PL 209 and PL 220. The γ-sitosterol, β-sitosterol and stigmasterol were the major sterols

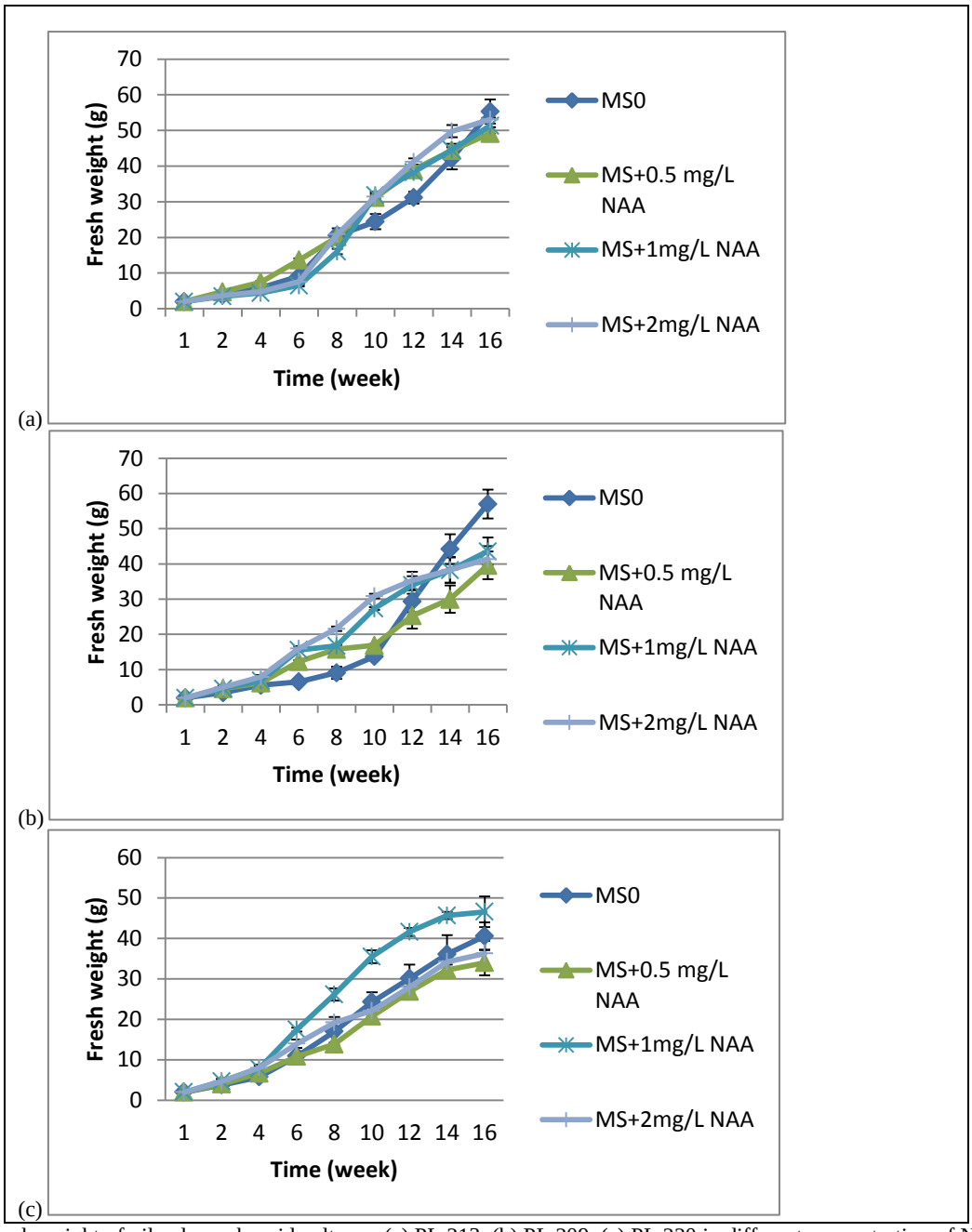

Fig 1. The fresh weight of oil palm embryoid cultures; (a) PL 213, (b) PL 209, (c) PL 220 in different concentration of NAA after 16 weeks.

present in all clones followed by stigmasterol, 22,23-dihydro-, stigmasta-7, 22-dien-3-ol, (3.beta.,5.alpha.,22Z)- and campesterol. Accumulation of sterol compounds in the extract of PL 213 embryoids was higher in type and percentage (Table 1) of each compound compared to their accumulation in the other two clones; PL 209 and PL 220. The sterol compounds that present in the extracts of PL 213 embryoid were γ-sitosterols, β-sitosterols, stigmasterol, campesterol, stigmasterol, 22,23-dihydro- and stigmasta-7, 22-dien-3-ol, (3.beta.,5.alpha.,22Z)-. The highest percentage of area (10.76%) was found as stigmasterol, 22,23-dihydro-, γ-sitosterol and β-sitosterol in the treatment of MS media supplemented with 0.5 mg L^{-1} NAA. However, MS media supplemented with 2.0 mg L^{-1} NAA produced the lowest type of sterol compound which is stigmasterol (0.39%). The sterol compounds that present in the extracts of PL 209 embryoid were β-sitosterols, stigmasterol and stigmasterol, 22,23-dihydro-. However, there was no sterol compounds found in the treatment of MS media supplemented with 1.0 mg L^{-1} NAA and MS media supplemented with 2.0 mg L^{-1} NAA. The sterol compounds that present in the extracts of PL 220 embryoid were γ-sitosterols, β-sitosterols and stigmasterol. However, there was no sterol compounds found in the treatment of MS media supplemented with 0.5 mg L^{-1} NAA and MS media supplemented with 2.0 mg L^{-1} NAA. It indicated that sterol compounds were not been produced by oil palm embryoids cultured in medium with high concentration of NAA. β-sitosterol, compesterol, stigmasterol, ergosterol and brassicasterol are the principle plant sterols (Bailey, 1964). Plant sterols are amphiphilic and occur as membrane constituents. Apart from their role in maintaining adequate function of the plant cell membranes, plant sterols also act as precursors of plant growth factors (Piironen et al., 2000). Steroidal compounds play an important

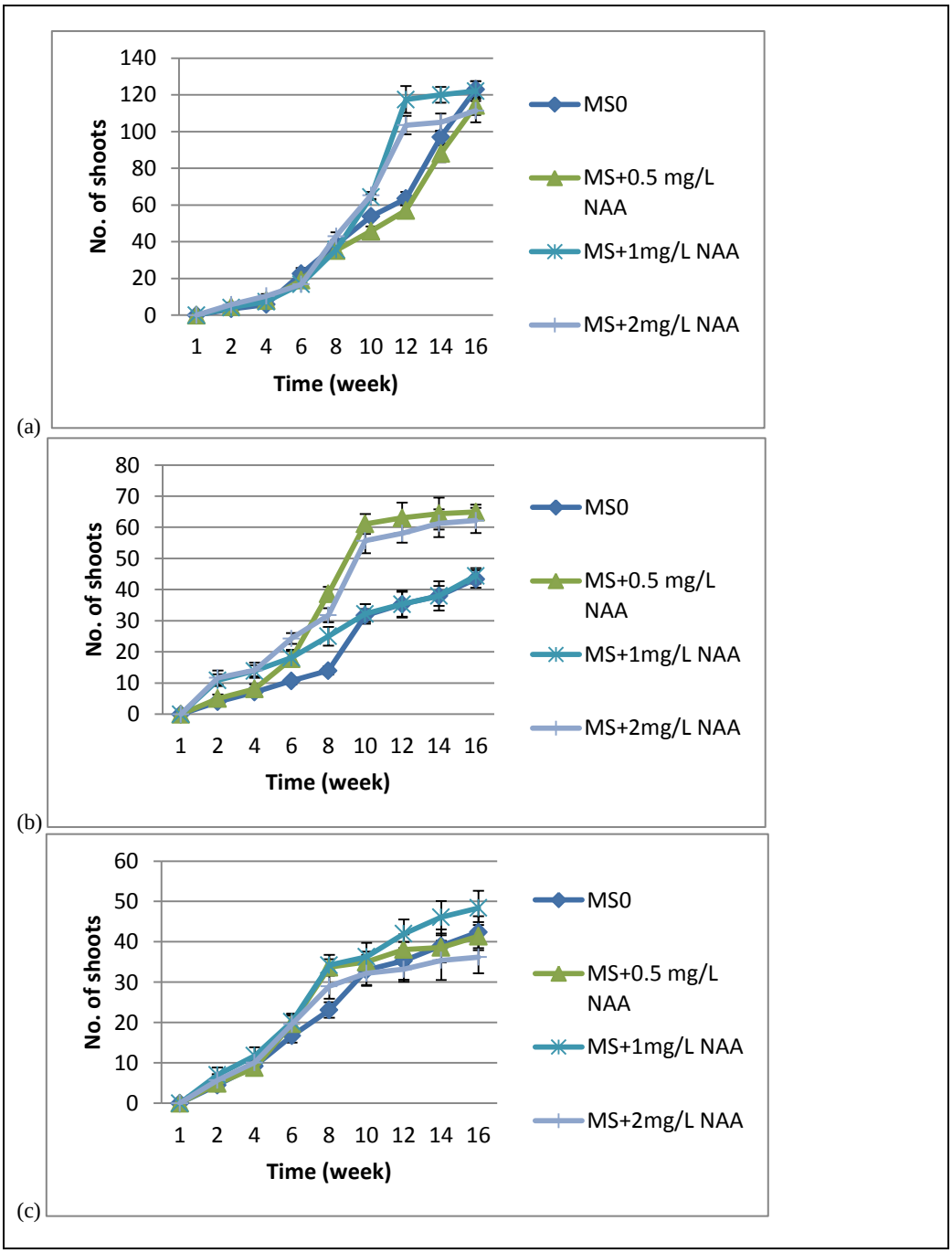

Fig 2. The number of shoots of oil palm embryoid cultures; (a) PL 213, (b) PL 209, (c) PL 220 in different concentration of NAA after 16 weeks.

role as plant defense metabolites apart from functioning as plant growth regulators (Faure et al., 2009) and regulating the fluidity of plant membranes for adaption to changes in temperature (Piironen et al., 2000). A previous study (Nusaibah et al., 2011) has shown that sterol compounds including β-sitosterol, γ-sitosterol, stigmasterol, campesterol and ergostenol were related to the oil palm defence mechanism against *Ganoderma boninense*. It indicates that the number and level of sterol compounds induced in infected palms were significantly higher than in uninfected seedlings. Another study by Defago and Kern (1983) using tomato, on its resistance to the pathogenic *Fusarium solani*, showed that higher levels of resistance towards fungal attacks were observed in tomato with a high sterol content in its membrane composition compared to that with a low sterol content.

Materials and Methods

Plant materials

In this study, callus-derived oil palm embryoids of three different Malaysian clones (PL 213, PL 209 and PL 220) have been used as the starting plant material. These Malaysian clones were established from oil palm liquid culture system (Tarmizi, 2002) by Malaysian Palm Oil Board (MPOB).

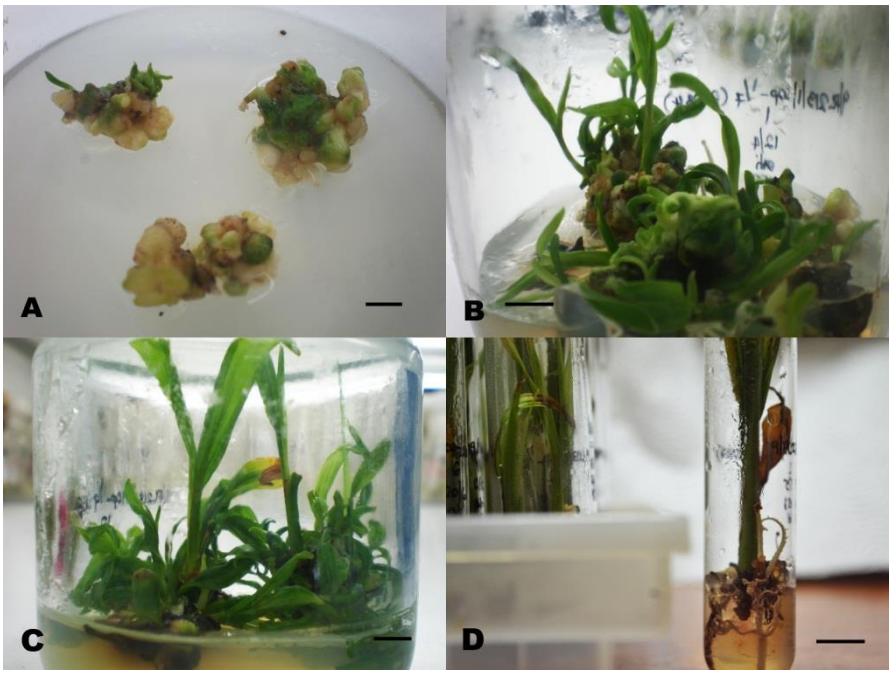

Fig 3. The development of PL 213 embryoids on MS medium without NAA (A) Eight-month early developed embryoids (B) Matured embryoids with shoots development after 8 weeks of culture (C) Shoots elongation after 16 weeks of culture (D) A rooted ramet produced after 16 weeks of culture. (A to D bar: 1 cm).

Preparation of culture medium

MS basal medium (Murashige and Skoog, 1962) was prepared with different concentrations of NAA (0, 0.5, 1.0 and 2.0 mg L^{-1}). The pH value was measured at 5.7-5.8 prior to autoclaving.

Multiplication of embryoids

Seven-month oil palm embryoids from clones PL 213, PL 209 and PL 220 were first been cultured on solid MS basal medium for a month in order to increase the biomass of yield prior to the NAA experiment. The cultures were incubated at 27 ± 2°C temperature and 12 hours light/day photoperiod.

NAA experiment

The experiment was then continued by culturing 2 g of embryoids to different treatments of MS media supplemented with several concentrations of NAA (0, 0.5, 1.0 and 2.0 mg L^{-1}) for growth and development. Ten replicates for each treatment have been set and the cultures were incubated at 27 ± 2°C temperature and 12 hours light/day photoperiod. Measurements on fresh weight (g), number of shoots and number of roots for all clones; PL 213, PL 209 and PL 220 were recorded every two weeks throughout the experiment. At the end of each experiment, the morphological changes of cultures were observed for the color and texture while the rate of embryoids maturation and germination were expressed as percentage. The growth rate was plotted by graph.

Extraction method

Oil palm embryoids were harvested after 16 weeks of culture. The fresh samples were dried in 50°C for five days, consecutively. Then, the oven dried and grounded samples were extracted using methanolic extraction as follows; 1 g of the sample was extracted twice in 10 ml of 100% MeOH for 24 hours at room temperature. The methanol extracts were then concentrated by rotary evaporation at 40°C after filtration (Grayer et al., 2008).

Identification of phytosterols in oil palm embryoids

GC-MS analysis

An Agilent 19091S-433 system was used to carry out GC-MS analysis. Chromatography was carried out with a HP-5MS capillary column (30 m long, 0.25 mm I.D. and a 0.25-µm 5%-phenylmethylpolysiloxane column). A standard 10-µl injection needle was mounted onto the auto sampler, and each sample (2 µl) was injected in split less mode. The carrier gas, helium, was at a flow rate of 1.5 ml min^{-1}; injector temperature at 280°C and detector temperature at 250°C.

Identification of the compounds

Identification of compounds present in the crude sample extracts was done by computer matching with the National Institute of Standards of Technology 02 (Nist 02) library data.

Statistical analysis

All results were expressed as mean ± S.D. Statistical analyses were performed by two-way analysis of variance (ANOVA) and Randomized Complete Block Design Analysis using SAS Software Version 9.00, with significance level at $p \leq 0.05$.

Conclusion

In conclusion, the highest growth rate and phytosterols production of oil palm (*Elaeis guineensis*) embryoid cultures was found in clones PL 213, followed by PL 209 and PL 220. All clones responded differently to the different NAA concentrations used in this study. GC-MS analysis has

enabled the identification of phytosterols present in oil palm embryoid extracts; with β-sitosterol, γ-sitosterol and stigmasterol being among the major sterol compounds detected. Further study can be done to find any correlation between phytosterols and oil palm defense mechanism.

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