Songklanakarin J. Sci. Technol.
33 (6), 653-657, Nov. - Dec. 2011

SJST SONGKLANAKARIN JOURNAL OF SCIENCE AND TECHNOLOGY
http://www.sjst.psu.ac.th

Original Article

Cytokinins and coconut water promoted abnormalities in zygotic embryo culture of oil palm

Komgrit Inpeuy, Suhaimine Chaemalee and Sompong Te-chato*

Department of Plant Science, Faculty of Natural Resources,
Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand.

Received 9 April 2011; Accepted 4 October 2011

Abstract

Induction of adventitious shoot formation from mature zygotic embryo of oil palm was carried out in liquid MS medium supplemented with various types of cytokinins. Kinetin (KN) alone at concentration of 0.5 mg/l gave the highest adventitious shoot formation at 13.4%. However, abnormal shoots in form of inflorescence-like structure (ILS) were obtained in 5 mg/l KN containing medium. For coconut water a big ILSs were formed at 10.6%. Histological studied revealed that those inflorescences had no clear floral organs.

Keywords: oil palm, zygotic embryo culture, abnormality, cytokinin, coconut water

1. Introduction

Several studies have been conducted to commercialize *in vitro* propagation of oil palm that could help to overcome the limitations associated with conventional asexual propagation techniques and to increase the number of plants produced in a short time. Two main sources of explants were chosen for the *in vitro* culture; young leaf (Ahee *et al.,* 1981; Pannetier *et al.,* 1981) and zygotic embryos (Teixeira *et al.,* 1993). In these two explants, embryo explants are more convenient because fruit are readily available, have a high degree of physiological uniformity, and can be shipped to a long distances. Recently, secondary somatic embryogenesis (SSE) was reported to be an efficient technique for plant propagation from both sources of explants (Hilae and Te-chato, 2007). Multiple shoot formation in *in vitro* culture is a disadvantage by using single shoot in this palm. Induction of embryo-derived shoots (EDS) through somatic embryogenesis is now recognized as a useful technique for propagation and *in vitro* conservation of oil palms. The whole processes of culture oil palms are no longer need cytokinin for shoot development from EDS or germination of somatic embryo (SE). Aberlence-Bertossi *et al.* (1999) observed that cultivating the cell suspensions for one month in a large quantity of liquid hormone-free medium, followed by plating the cells on BA-enrich medium, promoted the growth of proembryos, which then developed into globular and bipolar stage embryos.

Flowering involves complex developmental and physiological mechanisms and it is sensitive to environmental factors. The ability of explants to produce flowers *in vitro* depends upon various factors such as internal and external ones, as well as chemical and physical factors, which cannot be predicted. In terms of chemical factors, plant growth regulators play an important role in flowering. There have been some reports on *in vitro* flowering of orchids but it was species-dependent. Most of *in vitro* flowering orchids were stimulated by cytokinin (Kostenyuk *et al.,* 1999). Benzyladenine (BA) at 20 μM was effective for the promotion of flowering in the endangered species *Kniphofia leucocephala* Baijnath compared with isopentenyl adenine (2i-P) and zeatine (ZEA) (Taylor *et al.,* 2005; Nguyen *et al.,* 2006). Coconut water (CW) was found to be essential to trigger the formation of transitional shoot apical meristem and was found

* Corresponding author.
Email address: stechato@yahoo.com

to enhance inflorescence initiation and flower bud formation (Sim *et al.,* 2007). In case of rose cv. "First Prize", sucrose was reported to be the key factor in floral morphogenesis, while cytokinin increased the flowering percentage and helped the normal development of floral buds (Nguyen *et al.,* 2006).

So far, floral induction *in vitro* in oil palm tissue culture was not documented. Te-chato (2009) reported that flowering of oil palm was observed from tissue culture-derived plants at nursery stage. This event suggests that plant growth regulator (PGR) used for the tissue culture might affect the differentiation of floral organ after acclimatization to soil. Only few authors reported in vitro floral induction in date palm (Ammar *et al.,* 1987; Masmoudi-Allouche *et al.,* 2009). In these cases the authors indicated that photoperiod was a key factor in inducing both male and female flowers on 5-month-old seedlings. This paper reports the effects of BA, kinetin (KN) and CW in culture medium on abnormality of plantlet regeneration *in vitro* of oil palm.

2. Materials and Methods

Mature fruits of open pollinated oil palm were collected from various numbers of hybrid tenera (Dura x Pisifera; D x P) trees at Surat Thani Estate. The fruits were brought to the laboratory and mesocarp was completely removed from the fruits. The seeds were gently cracked and zygotic embryos surrounded by kernel were carefully removed using a sharp scalpel. The embryos with kernel were again trimmed to form a small cube of size 5x5x8 mm and used as explants for culture. The explants were surface sterilized by 70% ethyl alcohol for one minute and 20% Clorox for 20 minutes. Surface sterilant was removed from the explant by successive washing with sterile distilled water 3-5 times in a laminar flow station. The embryos were aseptically removed from kernel and cultured on culture medium without plant growth regulators.

2.1 Germination, adventitious shoot formation, and detection of flowering *in vitro*

Single shoots obtained by culturing mature zygotic embryo (MZE) on hormone-free MS medium for two months were decapitated and separated longitudinally into half segments. Each half was cultured in liquid MS medium supplemented with 3% sucrose and either 5 mg/l benzyl adenine (BA), 5 mg/l kinetin (KN), 0.5 mg/l thidiazuron (TDZ) or 15% CW. Each cytokinin or CW was used singly or in combination. All the liquid media were adjusted to pH 5.7 prior to autoclaving at 1.07 kg/cm^2, 120°C for 15 min. The cultures were maintained on rotary shaker at 100 rpm at 27±2°C under a 14–hr photoperiod with fluorescent lamps at 20 mmol/m^2/sec. After two months of culture viability and germination of ZE, multiple shoot formation, and flowering were recorded at monthly intervals.

2.2 Histological analysis

ILSs developed in liquid MS medium supplemented with 15% CW, 0.5 mg/l BA and 0.5 mg/l KN were collected and fixed in FAA II solution (formalin:glacial acetic acid:70% ethanol 5:5:90 v/v), dehydrated in ethanol-tertiary butanol series for 48 hrs and embedded in Parafin (Paraplast). Embedded tissues were sectioned with rotary microtome at 6-8 µm. The sections were stained with 'Fast Green' and Saffranin, and mounted with Permount on slide glass. Histological observation was carried out under blight field illumination of compound microscope.

2.3 Data analysis

For experimental design and statistical analysis, CRD with 4 replicates (each treatment was replicated twice and each replicate consist of 10 embryos/explants) was performed. The percentage of cultures that produced adventitious shoots or floral organ from different cross combinations was recorded every month after four months. The data were analyzed using analysis of variance (ANOVA) and mean among treatments was separated by Duncant's multiple range test (DMRT).

3. Results

Each shoot half cultured on solidified MS medium with all types and concentrations of PGR could not produce multiple shoots after three month of culture. Only one shoot was obtained from a half shoot explant (Figure 1). Different genotypes gave different responses. D174xP206 cultured with 5 mg/l BA or 5 mg/l KN survived at 100%, which was significantly different ($p<0.05$) from the other two genotypes (Table 1). Survived half shoot produced only one shoot with one to two leaves. Cultured half shoot in PGR-free medium turned brown at the highest percentage in all genotype tested (see Table 1). However, a slightly higher number of roots (0.25 root/shoot) was observed in this medium. Genotype D366xP110 gave the best response in shoot development with average leaf number of 2.1 leaves/explant when cultured

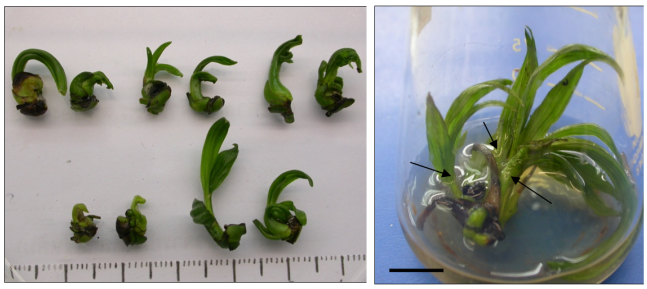

Figure 1. Development of single shoot (A) from cultured half shoot in solidified and adventitious shoots formation (B) in liquid MS medium for three months. (bar=1cm).

Table 1. Effect of PGRs on development of cultured half shoot on MS medium supplemented with 3% sucrose and 0.65% agar for three months.

Crosses	PGR (mg/l)	Survival rate (%)	No. of leaves/ shoot	No. of roots/ shoot	Browning percentage
D174XP206	PGR-free	61.9bc	1.3bc	0.0b	49.5a
	BA(5)	100.0a	1.4bc	0.1ab	18.1bc
	KN(5)	100.0a	1.6abc	0.0b	31.4abc
	TDZ(0.5)	54.3bc	1.0c	0.0b	29.5abc
	CW(150)[1]	79.1ab	2.1a	0.0b	18.1bc
D366XP110	PGR-free	68.9abc	1.4bc	0.3a	48.9a
	BA(5)	87.8ab	1.2bc	0.0b	42.2ab
	KN(5)	88.9ab	1.5bc	0.0b	11.1c
	TDZ(0.5)	61.1bc	1.0c	0.0b	31.1abc
	CW(150)[1]	74.4ab	2.1a	0.0b	17.8bc
D865XP110	PGR-free	65.6bc	1.2bc	0.1b	48.9a
	BA(5)	56.7bc	1.1c	0.0b	41.1ab
	KN(5)	67.8abc	1.3bc	0.0b	37.8abc
	TDZ(0.5)	37.8c	1.0c	0.0b	35.6abc
	CW(150)[1]	77.8ab	1.7ab	0.0b	23.2abc
F-test		*	*	*	*
C.V.(%)		24.5	21.3	312.6	43.4

[1] ml/l; * Significant difference at $p<0.05$; Mean sharing the same letters in common within column is significant difference by DMRT.

on CW-containing medium. The medium supplemented with 15% CW resulted in low frequency of browning percentage in average (Table 1).

By culturing half shoot in liquid medium of the same components supplemented with 15% CW, 0.5 mg/l BA and 0.5 mg/l KN for four months (subcultured at monthly intervals) adventitious shoot formation was obtained at 2.5 shoots/cultured half shoot (Figure 1). In addition, abnormalities of shoots characterized by the developed small or big size of inflorescent-like structure (ILS) were also found. A large number of small sized ILS (75%) was observed at 5 mg/l KN whereas big sized ILS (10.6%) was found in CW containing medium. Even though MS medium containing all the PGRs and CW resulted in the highest frequency of adventitious shoot formation (13.4%), they also caused ILS of both sizes (Table 2). Both sizes of ILS developed after two months of culture and look like hydrilla or water thyme (Figure 2A). After that the branching of ILS was well developed with clusters of floral buds (Figure 2B).

3.1 Histological analysis

Histological observation of this structure revealed that floral organs e.g. perianths, ovary occurred at auxiliary branches of inflorescence and apical portion (Figure 2C). Unfortunately, full development of those organs into complete flowers and bloom *in vitro* was not observed. This pheno-

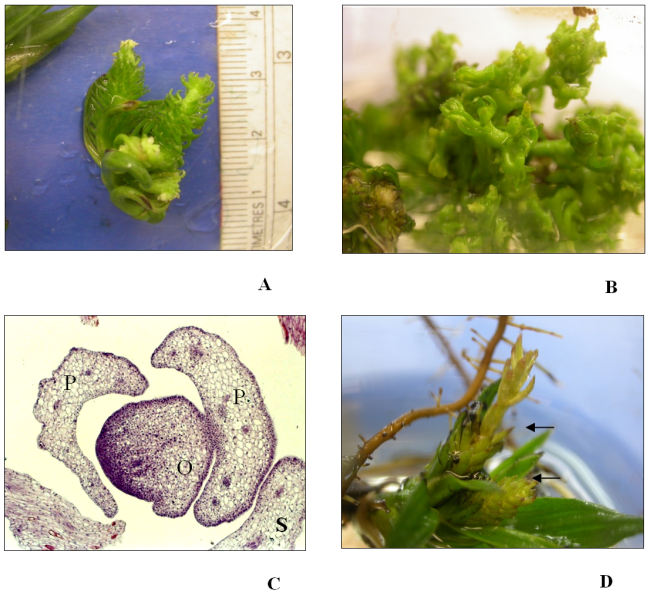

Figure 2. Abnormality of cultured half shoot in liquid MS medium supplemented with 15% CW, 0.5 mg/l BA and 0.5 mg/l KN after 4 months of culture. A: initiation of ILS after two months of culture. B: branching of floral bud on inflorescence axis. C: histology of floral bud revealed different whorl of floral organs. S: sepal, P: petal, O: ovary, D: development of big inflorescence after four months of culture.

Table 2. Effect of PGRs on ILS and adventitious shoot formation on liquidified MS medium supplemented with 3% sucrose after four months of culture.

PGRs (mg/l)	ILS formation (%)		Adventitious shoot (%)	No. of shoots/ explant
	Small size	Big size		
PGR-free	0.0c	0.0	0.0b	0.0b
KN(5)	75.0a	0.0	0.0b	0.0b
CW(150)[1]	0.0c	10.6	5.0ab	1.0ab
CW(150)[1]+BA(0.5)+KN(0.5)	13.4b	6.3	13.4a	2.5a
F-test	*	ns	*	*
C.V.(%)	2.9	102.5	73.5	77.4

[1] ml/l; ns: not significant difference; *: Significant difference at $p<0.05$; Mean sharing the same letters in common within column is significant difference by DMRT.

menon was also observed in a big size of floral bud (Figure 2D). These organs proliferated very well in liquid medium. After they were transferred to culture on agar-solidified medium of the same component with or without activated charcoal (AC), proliferation was arrested and further development of floral organ failed. ILS turned brown and died two months after transfer.

4. Discussion

Generally, cytokinin induces adventitious or multiple shoot formation in culturing shoot tips or axillay explants *in vitro* both in monocots and dicots. Although different plant species responds to different kind and concentration of cytokinin, BA was proved to be the most effective for that purpose (Liu and Li, 2001; Goh *et al.,* 1994). Normally, addition of cytokinin into solidified medium is enough to induce a large number of shoots (Arinaitwe *et al.,* 2000). In case of oil palm, there have been no reports on organogenesis through shoot tip culture. In most case single shoot was induced from both mature (Thawaro and Te-chato, 2009) and immature zygotic embryos (Srisawat and Kanchanapoom, 2005) in either liquid or solid medium. However, shaking culture of the embryos promoted a higher rate of absorption of water and mineral nutrients as well, lead to easy germination and development of seedling (Srisawat and Kanchanapoom, 2005). They also reported that culture of the embryos on solid medium caused the higher browning of the explant. Musgrave (1994) reported that cytokinin could prevent oxidation of the culture tissue giving rise to minimization of browning symptom and delay ripening of the fruits. CW contains cytokinins or cytokinin-like substances, which promote multiple shoot formation and popularly used in clonal propagation of orchid (Te-chato *et al.,* 2009) and banana (Gupta, 1986). In the present study, BA and other cytokinins were not effective in induction of multiple shoots whereas CW at 15% (v/v) promoted a healthy development of shoots with normal leaves.

Cytokinin is considered to be one of the most important physiological signals in flowering (Bernier *et al.,* 1993; Bonhomme *et al.,* 2000). In many plant species including orchids, addition of cytokinin to the culture medium was also found to induce early flowering *in vitro* (Bernier *et al.,* 1988; Peeters *et al.,* 1991). For example, in *Arabidopsis,* the addition of cytokinin iPA was effective in inducing early bolting and flower bud formation *in vitro* (He and Loh, 2002). The induction of vegetative shoot apical meristem to inflorescence meristem was observed when *Dendrobium* Madame Thong-In protocorms were cultured in modified liquid Knudson C medium (Knudson, 1946) containing BA and CW (Sim *et al.,* 2008). BA was reported to be metabolized easily into free radical of riboside which related to flowering in vitro of oil palm (Jones, 1998). Our previous study (Hilae and Te-chato, unpublished data) found that addition of BA at 5 mg/l to SE germination medium of oil palm resulted in an abnormality of shoots.

Acknowledgement

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Graduate School of Prince of Songkla University, the Oil Palm Agronomical Research Center (OPARC) of Southern Thailand and the Center of Excellence in Agricultural and Natural Resources Biotechnology.

References

Ahee, J., Arthuis, P., Cas, G., Duval, Y., Guenin, G., Hanower, J., Lievoux, D., Lioret, C., Malaurie, B., Pannetier, C., Raillot, D., Varechon, C. and Zuckerman, L. 1981. La multiplication vegetative in vitro du palmier a huile par embryogenese somatique. Oleagineux 36,113-125.

Bernier, G. 1988. The control of floral evocation and morphogenesis. Annual Review of Plant Physiology and Molecular Biology 39, 175-219

Bernier, G., Havelange, A., Houssa, C., Petitjean, A. and Lejeune, P. 1993. Physiological signals that induce flowering. Plant Cell 5, 1147–1155.

Bonhomme, F., Kurz, B., Melzer, S., Bernier, G. and Jacqmard, A. 2000. Cytokinin and gibberellin activate *SaMADSA*, a gene apparently involved in regulation of the floral transition in *Sinapis alba*. Plant Journal 24, 103-111.

Gupta, P.P. 1986. Eradication of mosaic disease and rapid clonal multiplication of bananas and plantains through meristem tip culture. Plant Cell, Tissue and Organ Culture 6, 33-39.

He, Y.W., Loh, C.S. 2002. Induction of early bolting in *Arabidopsis thaliana* by triacontanol, cerium and lanthanum is correlated with increased endogenous concentration of isopentyl adenosine (iPAdos). Journal of Experimental Botany 53, 505-512.

Jones, L. H. 1998. Metabolism of cytokinins by tissue culture lines of oil palm (*Elaeis guineensis* Jacq.) producing normal and abnormal flowering palms. Plant Growth Regulation 17, 205–214.

Jones, L. H., Hanke, D. E. and Eeuwens, C. J. 1995. An evaluation of the role of cytokinins in the development of abnormal inflorescences in oil palms (*Elaeis guineensis* Jacq.) regenerated from tissue culture. Plant Growth Regulation 14,135-142.

Knudson, L. 1946. A new nutrient solution for germination of orchid seed. American Orchid Society Bulletin 15, 214-217.

Musgrave, M. E. 1994. Cytokinins and oxidative processes. *In* Cytokinins: Chemistry, Activity, and Function (ed. W. S. M. David and C. M. Machteld). pp. 167-175. United States: CRC Press.

Nguyen, H.V., Phan, H.A and Duong, T.N. 2006. The role of sucrose and different cytokinins in the *in vitro* floral morphogenesis of rose (hybrid tea) cv. "First Prize". Plant Cell, Tissue and Organ Culture 87, 315-320.

Nwanko, B. A. and Krikorian, A. D. 1983. Morphogenetic potential of embryo and seedling derived callus of *Elaeis guineensis* Jacq. var pisifera Becc. Annals of Botany 51, 65-76.

Peeters, A.J.M., Gerards, W., Barendse, G.W.M., Wullems, G.J. 1991. *In vitro* flower bud formation in tobacco: interaction of hormones. Plant Physiology 97, 402-408.

Ravikumar, R., Ananthakrishnan, G., Kathiravan, K. and Ganapathi, A. 1998. *In vitro* shoot propagation of *Dendrocalamus strictus* nees. Plant Cell, Tissue and Organ Culture 52, 189–192.

Rhonda, L., Gambley and William A. D. 1990. An *in vitro* technique for the production de novo of multiple shoots in cotyledon explants of cucumber *(Cucumis sativus* L.). Plant Cell, Tissue and Organ Culture 20, 177-183.

Sim, G. Goh, C. and Loh, C. 2008. Induction of *in vitro* flowering in *Dendrobium* Madame Thong-In (Orchidaceae) seedlings is associated with increase in endogenous N^6-(2-isopentenyl)-adenine (iP) and N^6-(2-isopentenyl) -adenosine (iPA) levels. Plant Cell Reports 27, 1281-1289.

Srisawat, T and Kanchanapoom, K. 2005. The influence of physical conditions on embryo and protoplast culture in oil palm (E*laeis guineensis* Jacq.). ScienceAsia 31, 23-28.

Taylor, N.J., Light, M.E. and Van Staden, J. 2005. *In vitro* flowering of *Kniphofia leucocephala*: influence of cytokinins. Plant Cell, Tissue and Organ Culture 83, 327-333.

Te-chato, S., Nujeen, P. and Muangsorn S. 2009. Paclobutrazol enhance budbreak and flowering of Friederick's Dendrobium orchid *in vitro*. Journal of Agricultural Technology 5, 157-165.

Thomas, V. and Rao, P. S. 1985. *In vitro* propagation of oil palm (*Elaeis guineensis* Jacq. var. tenera) through somatic embryogenesis in leaf derived callus. Current Science 54, 184-185.