Journal of Agricultural Technology 2012 Vol. 8(4): 1485-1496
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Effect of bacteria density, inoculation and co-cultivation period on *Agrobacterium*-mediated transformation of oil palm embryogenic callus

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Sureerat Yenchon and Sompong Te-chato (2012) Effect of bacteria density, inoculation and co-cultivation period on *Agrobacterium*-mediated transformation of oil palm embryogenic callus. Journal of Agricultural Technology 8(4): 1485-1496.

The influence of density of *Agrobacterium*, inoculation and co-cultivation period on gene transformation in embryogenic callus of oil palm was investigated. *Agrobacterium* strain AGL-1 containing plasmid pCAMBIA1304 which carring *gus* and *hpt* as screenable and selectable marker genes, respectively, was used for transformation. The results showed that embryogenic callus at 4 weeks inoculated in *Agrobacterium* suspension at density of 0.8 (OD_{600}) for 6 hours and co-cultivated on solid MS medium with 200 µM acetosyringon in the dark for 3 days, followed by culturing on selective medium for 2 weeks gave the highest transient expression of *gus* gene at 100%. The hygromycin-resistant embryogenic callus was obtained at 63.89% and developed to form somatic embryos at 4 embryos/culture after 8 weeks of culture on selection medium. Polymerase chain reaction (PCR) analyse confirmed the presence of *gus* gene at size of 441 bps and *hpt* gene at size of 800 bps.

Key words: Agrobacterium, inoculation, co-cultivation, oil palm, embryogenic callus

Introduction

Oil palm (*Elaeis quineensis* Jacq.) is one of the most economically important sources of vegetable oil. It is a perennial monocotyledonous plant with a long generation period of about 20 years. Thus, oil palm breeding is a very slow process (Abdullah *et al.*, 2005). Plant genetic engineering is powerful tool to manipulate the genome of an organism for improving a new trait. There are several ways to introduce foreign genes into plants, for example, electroporation, microinjection, polyethyleneglycol transfer and particle bombardment (direct transformation) and *Agrobacterium*-mediated transfer (indirect transformation). *Agrobacterium*-mediated transformation method has most widely been used for various plant species due to its easy feasibility

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without any special equipment. Although monocotyledonous plants are not the natural host of *Agrobacterium,* recent development of technologies by the critical evaluation of the necessary factors enable us to apply *Agrobacterium*-mediated transformation to various monocotyledonous species such as lily (Ogaki *et al*., 2008), rice (Rachamawati *et al*., 2004), maize (Sidorov *et al*., 2006), sugarcane (Manickavasagum *et al*., 2004) and sorghum (Zhao *et al*., 2000). For oil palm, there are several reports on the trail to introduce foreign genes by partical bombardment (Chowdhury *et al*., 1997; Paveez *et al*., 1998; Abdullah *et al*., 2005; Lee *et al*., 2006). In case of *Agrobacterium*-mediated transformation, it has been reported by Abdullah *et al*. (2005). In their study immature zygotic embryos (IZE) were infected with *Agrobacterium* strain LBA4404 (pCAMBIA1301) for 30 min. The results showed *gus* expression at 64.4%. Therefore, it is now expected to establish efficient *Agrobacterium*-mediated transformation systems as an alternative approach to achieve such breeding for high yield, resistance to biotic and abiotic stress and improving the oil quality in oil palm. In this present study, we describe some biotic factor, density of *Agrobacterium* and physical factors, time of inoculation and incubation to enhance efficiency of genetransformation in embryogenic callus of oil palm.

Materials and methods

Plant material

Embryogenic callus (EC) initiated from young leaves of elite "Tenera" tree was cultured on callus induction medium (CIM) by the methods described by Te-chato *et al.* (2002). The cultures were maintained on MS medium supplemented with 3% sucrose, 200 mg/l ascorbic acid and 0.3 mg/l dicamba, and adjusted pH to 5.7 before adding agar and autoclaving at 1.05 kg/cm^2, 121ºC for 15 min. The cultures were placed at 28±2ºC under 14 hr photoperiod at 1,300 lux illumination and subcultured monthly intervals.

Preparation of Agrobacterium cells

Agrobacterium tumefaciens strain AGL-1 containing the plasmid pCAMBIA1304 which harbored *ß*-glucuronidase (*gus*) and hygromycin phosphotransferase (*hpt*) genes was used for inoculation in this study. A single colony of this bacteria was suspended in 30 ml liguid LB medium (10 g/l Tryptone, 5 g/l Bacto yeast extract, 5 g/l NaCl, pH 7.0) containing 50 mg/l kanamycin and proliferated on a rotary shaker at 150 rpm in the dark at 28 ^{o}C.

After proliferation in LB medium for 24 h the cell were collected and adjusted density by spectrophotometer at optical density (OD) of 600 nm.

Transformation procedure

The EC at 4 weeks was immersed in the bacterial suspension at four levels of cell densities (OD_{600}) i.e. 0.4, 0.6, 0.8 and 1.0 and inoculated for 2, 4, 6 and 12 hours on rotary shaker at 150 rpm. The excess of bacteria were removed from the EC by placing them on sterile filter paper before transfer to co-cultivation medium (solid MS medium contiaining 0.3 mg/l dicamba and 200 µM acetosyringon) and kept in the dark at 28 ^{o}C. The co-cultivation period was varied from 2 to 5 days. After co-cultivation, EC was washed with liguid MS medium containing 400 mg/l cefotaxime for 10 min and liquid MS free medium for 5 min. Then the EC was blotted on sterile paper and transferred to MS medium supplemented with 200 mg/l cefotaxime to eliminate bacteria for 2 weeks. EC was transferred to selective medium (MS medium contiaining 0.3 mg/l dicamba, 200 mg/l ascorbic acid and 30 mg/l hygromycin) to obtain transformed tissues. The cultures were placed under 1,300 lux illumination, 14 h photoperiod at 28±2ºC and subcultured 2 weeks intervals. The hygromycin-resistant EC were obtained after 8 weeks of culture on selection medium.

Histochemical assay for GUS activity

Two weeks after culture on selective medium, ECs (20-25 pieces) collected from each treatment were subjected to transient histochemical GUS assay (Jefferson *et al*, 1987). EC were incubated in sodium phosphate buffer (50 mM) containing 5-bromo-4-chloro-3-indolyl *ß*-D-glucuronide (X-Gluc) as the substrate overnight at 37 ºC. The stained EC were soaked in 70% methyl alcohol to remove the chlorophyll from explant tissue. Transformation efficiency was evaluated by the number of callus expressing blue spots in comparison with total number of EC.

Polymerase chain reaction (PCR) analysis

Genomic DNA was isolated from EC (0.05 g) after 8 weeks of culture on selective medium by the protocol described by Te-chato (2000). To detect the presence of *gus* and *hpt* genes genomic DNA was amplified by *gus* primer (F-primer 5'-CTGCGACGCTCACACCGATAC-3' and R-primer 5'-TCACCGAAGTTCATGC CAGTCCAG-3') and *hpt* primer (F-primer 5'-CCTGAACTCACCGCGACG-3' and R-primer 5'-AAGACCAATGCGGA GCATATA-3'). PCR products were then run on 1.5% agarose gel at 100 V and

detected by ethidium bromide staining. The product sizes of both genes were 441 bps for *gus* gene and 800 bps for *hpt* gene.

Statistical analysis

Data of mean were analyzed using a one-way analysis of variance (ANOVA). Significant differences among treatments were detected using Duncan's multiple range tests (DMRT) at the 0.01 or 0.05 level of probability.

Results

Effect of density of Agrobacterium, inoculation and co-cultivation period on expression of gus gene

A tri-factorail experiment was designed using four levels of *Agrobacterium* cell density (OD_{600}) at 0.4, 0.6, 0.8 and 1.0, four levels of inoculation period i.e., 2, 4, 6 and 12 h, and three levels of co-cultivation i.e., 2, 3 and 5 days. Transient *gus* expression was observed in inoculated EC after culture on selective medium for 2 weeks. The results showed that embryogenic callus at 4 weeks after inoculation with *Agrobacterium* suspension at density of 0.8 for 6 hours and co-cultivated on solid MS medium with 200 μM acetosyringon in the dark for 3 days, followed by culturing on selection medium for 2 weeks gave the highest transient expression of *gus* gene at 100% significant difference to other treatments (Table. 1, Fig. 1).

Table 1. Effect of densities of *Agrobacterium*, inoculation and co-cultivation period on transient *gus* expression of EC after culture on selective medium for 2 weeks

Density of bacteria (OD_{600})	Inoculation period (h)	*gus* expression (%) Co-cultivation period (days)			Mean Inoculation	Mean Density
		2	3	5		
0.4	2	11.43g	24.44fg	25.72fg	20.53C	56.43C
	4	50.00bcdef	28.89efg	46.67defg	41.85BC	
	6	68.89abcd	100.00a	91.67ab	86.85A	
	12	48.89cdefg	91.67ab	88.89abc	76.48AB	
0.6	2	66.67abcde	80.56abcd	72.22abcd	73.15AB	78.47B
	4	61.67abcdef	88.89abc	72.22abcd	74.26AB	
	6	72.22abcd	100.00a	91.67ab	87.96A	
	12	55.00bcdef	88.89abc	91.67ab	78.52AB	
0.8	2	61.67abcdef	91.67ab	72.22abcd	75.19AB	86.25A
	4	86.67abcd	100.00a	83.33abcd	90.00A	
	6	91.67ab	100.00a	91.67ab	94.45A	
	12	75.56abcd	100.00a	80.56abcd	85.37A	
1.0	2	63.89abcdef	75.00abcd	83.33abcd	74.07AB	81.76AB
	4	55.56bcdef	91.67ab	71.67ab	73.62AB	
	6	63.89abcdef	100.00a	91.67ab	85.19A	
	12	86.67abcd	88.89abc	88.89abc	88.15A	
Mean Co-culture		63.77B	84.41A	79.00A		

Means not sharing letters in common are significantly different by DMRT
Different capital letters indicate significant differences among treatments at $p \leq 0.001$.
Different small letters indicate significant differences among treatment combinations (density of *Agrobacterium*, inoculation period and co-cultivation period) at $p \leq 0.001$.

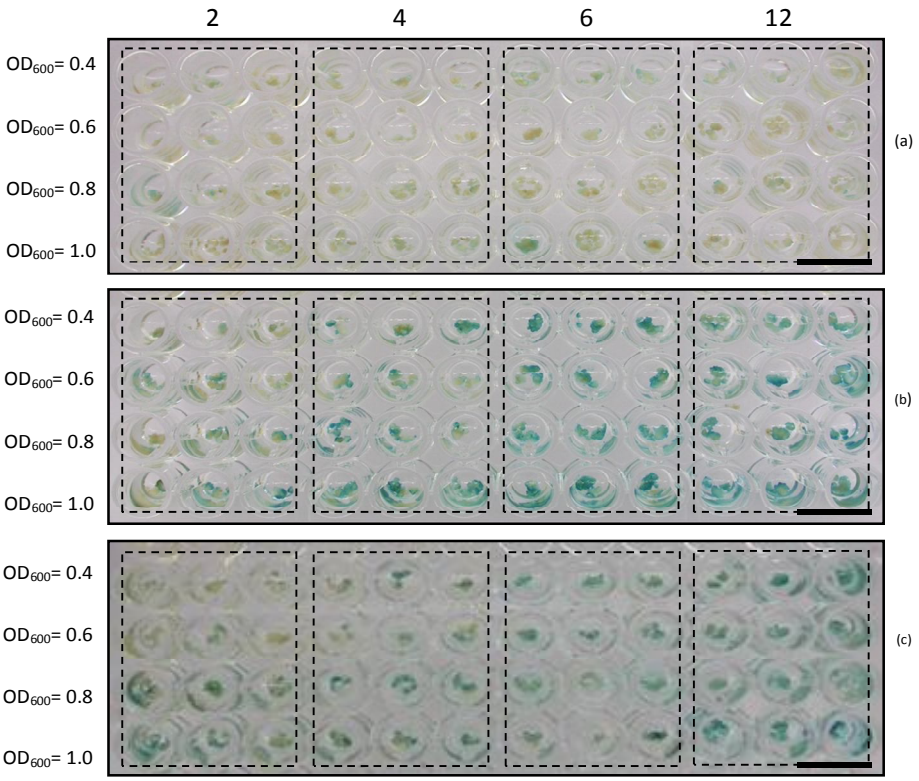

Fig. 1. Transient *gus* expression in EC after 2 weeks of selection. (bar=1 cm) (a), (b), (c) ; co-cultivated for 2, 3 and 5 days.

Effect of densities of Agrobacterium, inoculation and co-cultivation period on hygromycin-resistant EC

The embryogenic callus at 4 weeks after inoculation with *Agrobacterium* suspension at density of 0.8 for 6 hours and co-cultivated on solid MS medium with 200 µM acetosyringon in the dark for 3 days gave the highest hygromycin-resistant EC at 63.89% and developed to form somatic embryos at 4 embryos/culture after 8 weeks of culture significant difference to other treatments (Table 2, 3 and Fig. 2).

Table 2. The effect of density of *Agrobacterium*, inoculation and co-cultivation period on hygromycin-resistant EC after culture on selective medium for 8 weeks

Density of bacteria (OD_{600})	Inoculation period (h)	Hygromycin-resistant EC (%) Co-cultivation period (days)			Mean Inoculation	Mean Density
		2	3	5		
0.4	2	0.00c	0.00c	0.00c	0.00D	16.44B
	4	11.11abc	11.11abc	8.33bc	10.18CD	
	6	33.33abc	52.78abc	11.11abc	32.41ABC	
	12	19.44abc	38.89abc	11.11abc	23.15BCD	
0.6	2	30.55abc	41.67abc	22.22abc	31.48ABC	36.11A
	4	30.55abc	41.67abc	19.44abc	30.55ABCD	
	6	41.67abc	55.56ab	63.89a	53.71AB	
	12	22.22abc	52.78abc	11.11abc	28.70ABCD	
0.8	2	41.67abc	30.55abc	30.56abc	34.26ABC	36..11A
	4	22.22abc	61.11ab	22.22abc	35.18ABC	
	6	52.78abc	63.89a	52.78abc	56.48A	
	12	22.22abc	22.22abc	11.11abc	18.52DC	
1.0	2	22.22abc	22.22abc	33.33abc	25.92ABCD	31.48A
	4	41.67abc	41.67abc	30.56abc	37.97ABC	
	6	41.67abc	41.67abc	22.22abc	35.19ABC	
	12	30.55abc	30.56abc	19.44abc	26.85ABCD	
Mean Co-culture		28.99B	38.02A	23.09B		

Means not charring letters are significantly different by DMRT
Different capital letters indicate significant differences among treatments at $p \leq 0.001$.
Different small letters indicate significant differences among treatment combinations (density of *Agrobacterium*, inoculation period and inoculation period) at $p \leq 0.001$.

Table 3. Effect of densities of *Agrobacterium*, inoculation and co-cultivation period on somatic embryo formation after culture on selective medium for 8 weeks

Density of bacteria (OD_{600})	Inoculation period (h)	No. of SE/tube Co-cultivation period (days)			Mean Inoculation	Mean Density
		2	3	5		
0.4	2	0.00d	0.00d	0.00d	0.00C	0.80B
	4	0.33dc	0.33dc	1.00abcd	0.55BC	
	6	1.17abcd	1.56abcd	1.00abcd	1.24BC	
	12	1.33abcd	1.83abcd	0.67bcd	1.28BC	
0.6	2	1.33abcd	1.83abcd	1.00abcd	1.39BC	1.76A
	4	1.00abcd	1.50abcd	1.00abcd	1.17BC	
	6	2.17abcd	1.83abcd	3.00abcd	2.33AB	
	12	2.67abcd	3.5ab	0.33dc	2.17AB	
0.8	2	1.33abcd	1.67abcd	1.00abcd	1.33BC	1.84A
	4	0.5bcd	3.06abcd	1.33abcd	1.63ABC	
	6	2.83abcd	4.00a	3.17abc	3.33A	
	12	0.5bcd	2.00abcd	0.67bcd	1.06BC	
1.0	2	1.33abcd	1.00abcd	1.00abcd	1.11BC	1.32AB
	4	1.33abcd	2.00abcd	1.17abcd	1.50BC	
	6	1.5abcd	2.67abcd	1.00abcd	1.84ABC	
	12	1.00abcd	0.83bcd	1.00abcd	0.94BC	
Mean Co-culture		1.27B	1.85A	1.17B		

Means not charring letters are significantly different by DMRT
Different capital letters indicate significant differences among treatments at $p \leq 0.001$.
Different small letters indicate significant differences among treatment combinations (density of *Agrobacterium*, inoculation period and inoculation period) at $p \leq 0.001$.

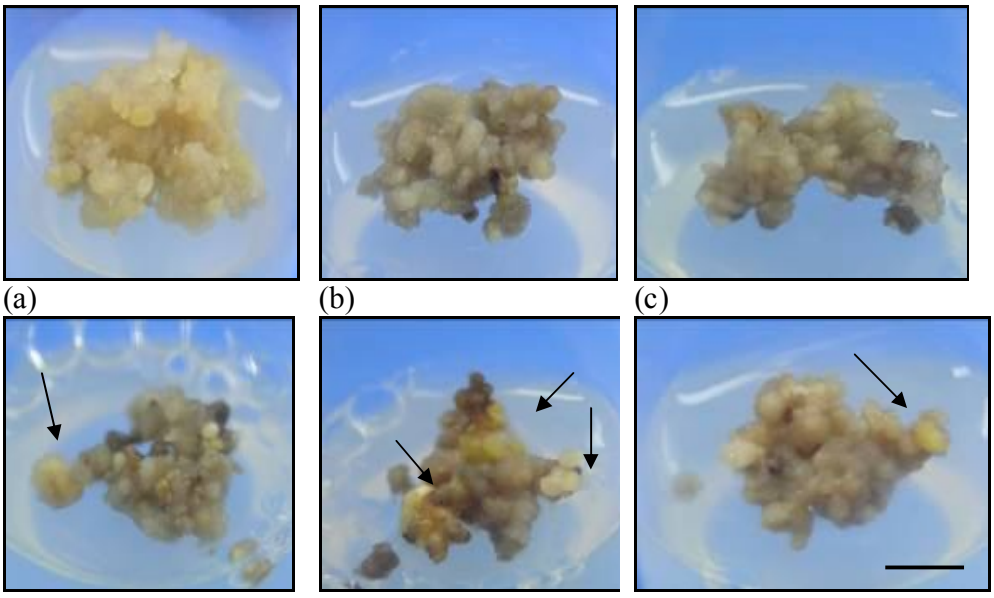

Fig. 2. Hygromycin-resistant EC and SE formation (arrows) obtained from co-cultivation the callus with *Agrobacterium* for 3 days and cultured on hygromycin containing medium for further 8 weeks (bar=2.5mm).
(a); control (without hygromycin) (b); control (with hygromycin) (c)–(f); 2, 4, 6, 12 h inoculation period, respectively.

Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) analyse confirmed the presence of *gus* gene at size of 441 bps and *hpt* gene at size of 800 bps from transformed EC. Both of those sizes of DNA (gene) didn't appear from controlled EC (Fig. 3).

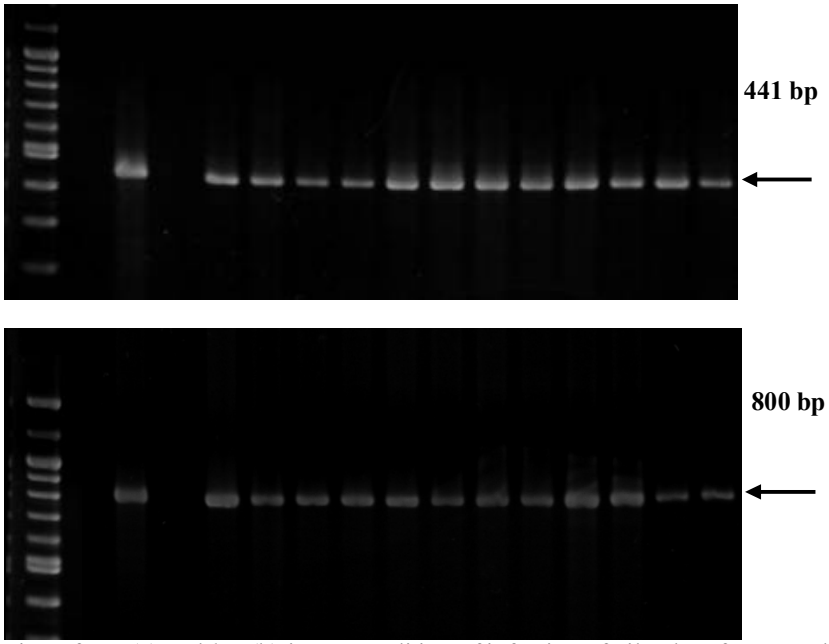

Fig. 3. Detection of *gus* (a) and *hpt* (b) in EC condition of infection of oil palm after 8 weeks of selection by PCR.
M: DNA marker N: negative control P: positive control 1-12: transformed EC

Discussion

In this study, influence of *Agrobacterium* density during inoculation and co-cultivation at different period was investigated. The level of transient *gus* expression and hygromycin-resistant EC was highest when OD_{600} of bacterial cells at 0.8. The same density of *Agrobacterium* was successfully used with the winter jujube (Gu *et al*., 2008); sugarcane (Joyce *et al*., 2010) and *Acacia crassicarpa* (Yang *et al*., 2008). Nevertheless, higher or lower *Agrobacterium* densities were successfully used in another plant such as *Vigna radiata* at OD_{600}= 1.0 (Tazeen and Mirza, 2004); lily at OD_{600}= 0.6 (Ogaki *et al*., 2008) and tomato at OD_{600}= 0.1 (Gao *et al*., 2009). With higher or lower *Agrobacterium* densities, both transient *gus* expression and hygromycin-resistant EC were decreased. Because of *Agrobacterium* at high density usually damaged the plant cell, and resulted in lower cell recovery that ultimately reduced the transformation frequency (Cheng *et al*., 2004). Inoculation period is an important factor in *Agrobacterium* -mediated transformation. Transeint *gus* expression was increased with the period of inoculation. In many plants inouclation period only 15 – 30 min was reported, such as *Withania somnifera* (Pandey *et al*., 2010); rice (Nazim-Ud-Dowla *et al*., 2008). However, this present study found that inoculation for 6 h gave the best result. Give support

reason, duration of co-cultivation periods of the explants with *Agrobacterium* is an important factor as well which was found to influence genetic transformation. Generally, co-cultivation period varied from 3-5 days. In this study it was found that co-cultivation for 3 days was optimal for embryogenic callus of oil palm. Similar results were reported in transformation of *Vigna radiate* (Tazeen and Mirza, 2004); rice (Hoque *et al*., 2005; Arockiasamy and Ignacimuthu, 2007); pineapple (Gangopadhyay *et al*., 2009). However, co-cultivation for long time (5-6 days) was used in blueberry (Song and Sink, 2004) and *Withania somnifera* (Pandey *et al*., 2010). But in this study, co-cultivation for long time was reduced the transformation frequency. In addition, overgrowth of *Agrobacterium* was observed. This reason caused the reduction of cell recovery that difficult to regeneration into plantlet. Thus, co-cultivation plays important role in successful for *Agrobacterium*-mediated transformation of embryogenic callus of oil palm.

Acknowledgements

The authors are grateful to the Faculty of Natural Resources, the National Research University Project of Thailand, and the Graduate School of Prince of Songkla University and the Oil Palm Agronomical Research Centre of Southern Thailand and for financial support. This research is partially supported by the Centre of for Agricultural Biotechnology, Postgraduate Education and Research Development Office, Commission on Higher Education.

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(Published in July 2012)