Journal of Agricultural Technology 2008, V.4(2): 137-146

Induction of somatic embryogenesis and plantlet regeneration from cultured zygotic embryo of oil palm

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Chehmalee, S. and Te-chato, S. (2008). Induction of somatic embryogenesis and plantlet regeneration from cultured zygotic embryo of oil palm. Journal of Agricultural Technology 4(2): 137-146.

Plantlet regeneration through somatic embryogenesis of oil palm (*Elaeis quineensis* Jacq. D×P) was induced from culturing mature zygotic embryos (MZE). Somatic embryos (SEs) at the final stages called haustorium embryos (HEs) was obtained from 865(D)×110(P). The highest HEs producing embryogenic callus was obtained at 14 % from primary callus (PC) after culture on Murashige and Skoog (MS) medium supplemented with 1 mg/l 3,6-dichloro-2-methoxybenzoic acid (dicamba) in the presence of 200 mg/l ascorbic acid (As). In contrast, the highest percentage of embryogenic callus (EC) (26.03) was obtained in 174(D)×206(P) subsequent to increasing in fresh weight on MS medium with dicamba at concentration of 0.5 mg/l. Secondary somatic embryos (SSEs) were achieved at 80 % on MS medium supplemented with 0.2 M of sorbitol and 200 mg/l As. Plantlet conversion rate at the highest percentage of 3.7 was obtained from torpedo-stage SSEs on PGR-free MS medium for 3 months. Those complete plantlet could be readily excised and transferred to soil.

Key words: somatic embryogenesis, oil palm (*Elaeis quineensis* Jacq.), zygotic embryo, secondary somatic embryo

Introduction

Oil palm is one of the most economically important crops in the world. Cultivation of oil palm has expanded tremendously in recent years such that it is now second only to soybean as a major source of the world supply of oils and fats (Wahid *et al.*, 2004). Interest in palm oil as a biofuel could eventually cause constraints on worldwide supply of edible palm oil and increase the pressure for higher yield and/or cultivatable areas (Biofuel, 2007). The oil palm has only a single growing point, and does not produce suckers like some other palm species, so clones cannot be produced by the common techniques such as cutting, grafting or layering (Corley and Tinker, 2003). So it is possible to

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enhance efficiency for propagation through somatic embryogenesis, especially *in vitro* culture through zygotic embryo (ZE) culture and also embryo explants are convenient because fruits are readily available, have a high degree of physiological uniformity, and can be shipped long distances. The establishment of plant regeneration in oil palm by somatic embryogenesis is satisfactory, including ZE culture (Te-chato, 1998; Kanchanapoom and Domyoas, 1999). Various stages of ZE and genotypes of embryos were reported to be success by inducing somatic embryogenesis (Chehmalee and Te-chato, 2007). Dicamba has been reported to be an effective auxin for increasing a large number of somatic embryos (SEs) (Te-chato *et al.*, 2003). Regeneration of oil palm through secondary somatic embryos (SSEs) has also been reported using polyamines (Rajesh *et al.*, 2003). However, percentage and number of new forming embryos were limited and germination of those embryos were not reported. Sorbitol were reported to induce SSE from haustorium embryo (HE) derived from culturing young leaf of mature oil palm subsequent to a high frequency of plantlet formation (Te-chato and Hilae, 2007). So far, there have no reports on SSE formation in ZE culture. This project was to investigate the induction of SSE from ZE-derived HE subsequent to plantlet regeneration.

Materials and methods

Plant material

Mature fruits of *Elaeis quineensis* Jacq. hybrid Tenera were kindly provided by Klong Hoi Khong Research Station, Prince of Songkla University, Hat Yai, Songkhla, Thailand. MZE from three crosses between 'Dura' and 'Pisifera'; 174(D)×206(P), 865(D)×110(P) and 366(D)×110(P) were excised by the following protocol. The mesocarp was completely removed from fruits. The seeds were gently cracked and the embryos surrounded by kernel were carefully trimmed to form a small cube of size 5x5x8 mm^3 and used as explants for culture. The cubes were surface sterilized by 70% alcohol for 1 minute and 20% Clorox (containing 0.5 ml of Tween-20 emulsifier per 100 ml solution) for 20 minutes, followed by successive washing with sterile distilled water 3 times in laminar flow station. The embryos were aseptically removed from kernel and cultured on culture medium.

Establishment of embryogenic callus (EC)

PCs were established following the method described by Chehmalee and Te-chato (2007). PC induced from MZE of three crosses; 174(D)×206(P),

865(D)×110(P) and 366(D)×110(P) was regularly transferred to fresh MS medium monthly interval. The medium was supplemented with 1 mg/l dicamba, 200 mg/l As, 3% sucrose and gelled with 0.75% agar-agar. Each experiment was performed with 5 replicates. Each replicate consisted of 10 test tubes (25×150 mm containing 10 ml of medium). The pH of medium was adjusted to 5.7 prior autoclaving at 1.07 kg/cm^2 at 121^{o}C for 15 min. Cultures were maintained at 28±0.5ºC under 14 h photoperiod at 1,300 lux illumination and subcultured monthly intervals. The development of the callus and embryogenic callus, in terms of percentage and type were recorded after 3 months of culture.

Proliferation of embryogenic callus (EC)

ECs (approximately 50 mg) of the cross 174(D)×206(P) were carefully separated and inoculated on MS medium containing dicamba at concentrations of 0.1, 0.25, 0.5, 0.75 or 1 mg/l. Each concentration containing 200 mg/l As, 3% sucrose and gelling with 0.75% agar-agar. There were four replicates. Each replicate consisted of 5 test tubes. The culture was maintained under the same conditions as described in establishment of EC and subcultured monthly intervals. The fresh weight was recorded in every months after culture for 3 months.

Induction of secondary somatic embryo (SSE)

HEs of the cross 865(D)×110(P) were carefully detached and inoculated on hormone-free MS medium with 3% sucrose or replacing sucrose with 0.2 M sorbitol. Each media supplemented with 200 mg/l As and gelled with 0.75% agar-agar. There were 3 replicates, each containing 10 HEs/replicate. The culture was maintained under the same conditions as described earlier. The number of SSE/HE was counted and percentage of SSE per tube was recorded after 1 months of culture.

Germination of SSE

SSE from haustorium-staged embryos at 1, 2 and 3 months after maintenance on sorbitol containing medium were transferred to growth regulator-free MS medium supplemented with 3% sucrose solidified with 0.75% agar-agar. All cultures were carried out in bottle (60×110 mm containing 25 ml of medium) under the same conditions as described earlier. There were 3 replicates, each containing 10 SSEs/replicate. Germination

percentage in term of shoot and root production was recorded after 3 months of culture.

Data analysis

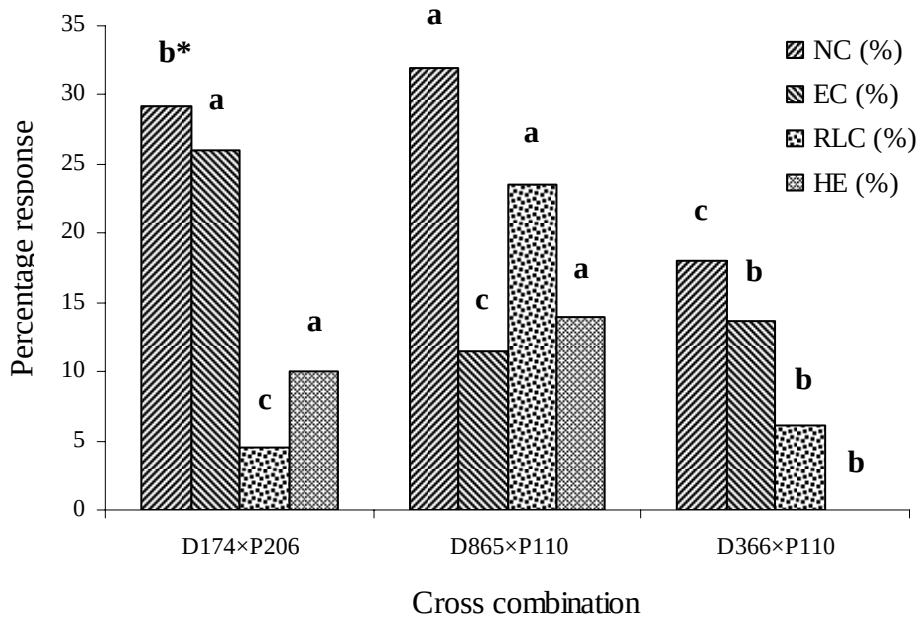
Data were analysed by ANOVA. Means were separated with Duncan's multiple range tests (DMRT) and Least significant difference (LSD) at the 0.01 and 0.05 level of probability, respectively. Where the F-test showed significant differences among means.

Results and discussion

Establishment of embryogenic callus (EC)

PC of three crosses were initiated from zygotic embryo of oil palm by using 2.5 mg/l dicamba after 3 months of culture following the method described by Chehmalee and Te-chato (2007). After 3 months of culture on MS supplemented with 1 mg/l dicamba in the presence of 200 mg/l As, various cross combinations gave the different response on callus formation (Fig. 1). Three types of callus were obtained from three different crosses. One is nodular callus (NC), second is embryogenic callus (EC) and finally root-like callus (RLC). Among those crosses, hybrid between 865(D) and 110(P) produced the best result in compact nodular callus formation at high frequency of 31.98% (Fig. 2A). Cross 174(D)×206(P) gave the highest EC formation (26.03%) which contained many small pearly white globular structure (Fig. 2B) followed by 366(D)×110(P) and 865(D)×110(P), respectively. In case of root like callus (RLC), 23.56% were obtained from cross 865(D)×110(P). This callus was elongate and succulent appeared at surface of the callus (Fig. 2C). For EC some of somatic embryo developed beyond globular type to haustorium stage. HEs developed from both 865(D)×110(P) (14%) and 174(D)×206(P) (10%), were greenish in color and separate as individual embryo (Fig. 2D) whereas cross 366(D)×110(P) fail to produce HE. Similar result was obtained in culturing immature inflorescence of peach palm. Crossings or genotypes play important role in somatic embryogenesis (Steinmacher *et al.*, 2007). Different varieties result in the different response on SE formation and plantlets development (Karun *et al*., 2004). Sarasan *et al*. (2005) also reported that genotype of the selected explants have influenced upon the type and percentage of callus formation. Moreover, in our previous study, the larger seeds consisted of larger size of ZE of all crosses gave the

higher percentage of germination and callus formation (Chehmalee and Te-chato, 2007).



* Value followed by different letter in term of type of callus are significantly different according to DMRT-test at P<0.01 level.

Fig. 1. Effect of cross combinations on callus (NC; nodular callus, EC; embryogenic callus RLC; root like callus) and haustorium embryo (HE) formation after culturing PC on MS medium supplemented with 1 mg/l dicamba and 200 mg/l As for 3 months.

Proliferation of embryogenic callus (EC)

Fresh weight of EC from the cross 174(D)×206(P) was gradually increased by increasing time of culture. After 3 months of culture, MS medium supplemented with 0.5 mg/l dicamba gave the best response on EC proliferation (Fig. 3) followed by dicamba at concentration of 0.25, 0, 0.1, 0.75 and 1 mg/l, respectively. The highest SE formation were obtained from MS medium supplemented with 0.1 mg/l dicamba (data not shown). Dicamba was found to be the best auxin for *in vitro* mass propagation of both seedling and mature oil palm (Te-chato *et al*., 2003). In addition, embryoids developed on medium containing 0.1 mg/l dicamba was found to be superior in inducing early stage of embryoid subsequent to further development of mature or haustorium embryoids (Te-chato, 1998). Some authors reported that low concentration of dicamba promoted somatic embryogenesis from immature

inflorescence (Steinmacher *et al*., 2007). Similar result was obtained in callus culture of *Areca catechu*. Decrease in concentration of dicamba stimulated proliferation rate of EC and also promoted a large number of embryoid formation (Wang *et al*., 2006).

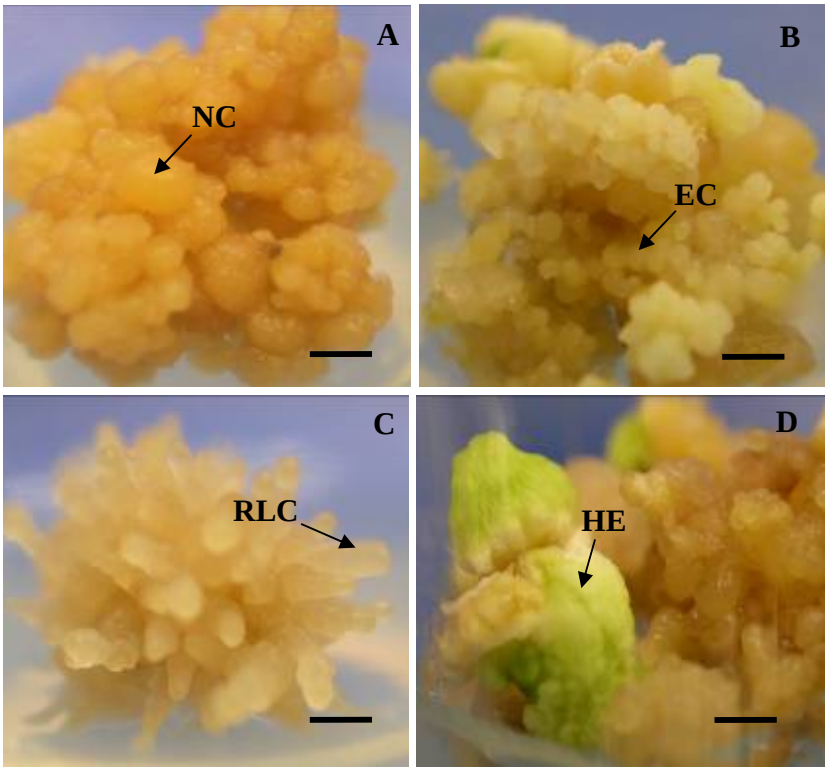


Fig. 2. Different types of callus obtained from various cross combinations after culturing PC on MS medium supplemented with 1 mg/l dicamba and 200 mg/l As for 3 months. A; Nodular callus (NC) from D865×P110 (bar: 2.75 mm), B; Embryogenic callus (EC) from D174×P206 (bar: 2.85 mm), C; Root like callus (RLC) (bar: 2.25 mm) and D; Haustorium embryo (HE) from D865×P110 (bar: 2.95 mm)

Induction of secondary somatic embryo (SSE)

SSE is a new cycle of somatic embryogenesis which could be induced directly from HE. For MS medium replacement sucrose with 0.2 M sorbitol in the present of 200 mg/l As gave better result in SSE formation (80%) and number of SSE/HE (15.81) than MS medium without sorbitol (20% and 4.55 SSE/HE, respectively) after one month of culture (Table 1). Prolong culture of HE in sorbitol containing medium produced more SSE (data not shown). And

also obtained that Full-strength MS medium supplemented with 0.2 M sorbitol produced significantly higher percentage and number of SSEs (Te-chato and Hilae, 2007). Polyamines were also reported to induce SSE from ZE culture of oil palm, however, percentage and number of new forming embryos still low (Rajesh *et al*., 2003). So, sorbitol was proved to be a better stress agent (water stress) than polyamine in promoting SSE both percentage and number. In this experiment, we found that SSE were induced from the basal part of HE. Those SSEs were cluster with white opaque characteristic consisted of torpedo-stage embryos (Fig. 4A). Promchan and Te-chato (2007) reported that SSE arose directly from epidermal layer at the basal part of HE.

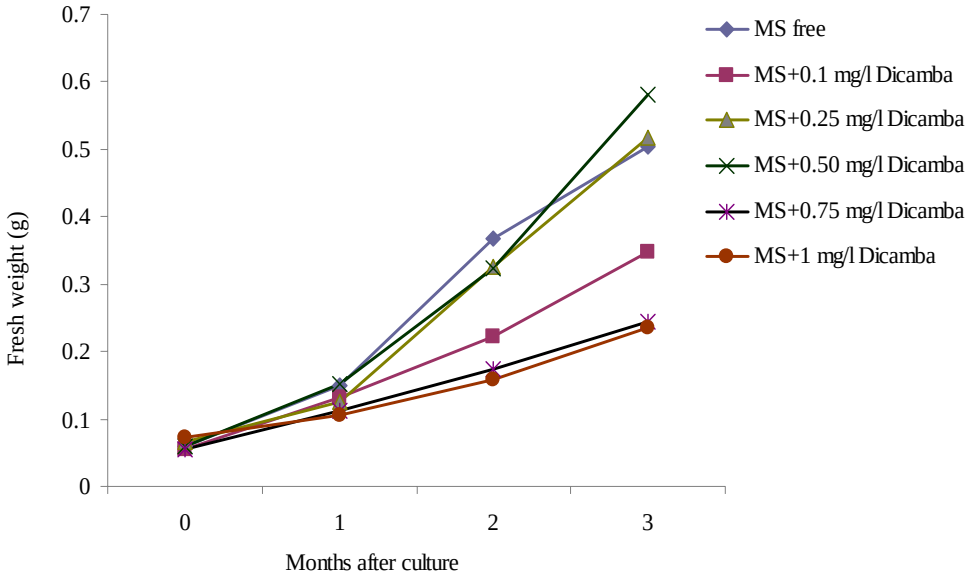


Fig. 3. Effect of various concentrations of dicamba on fresh weight of EC after culturing EC on MS medium in the presence of 200 mg/l As for 3 months.

Table 1. Effect of sorbitol on SSE formation after culturing HE on MS medium supplemented with 200 mg/l As for 1 month.

Sorbitol (M)	No. of HE	SSE formation	
		Percentage	Average number/HE
0 [1]/	30	20.00	4.55
0.2	30	80.00	15.81
Lsd.05		22.67	2.75
CV(%)		20.00	11.92

[1/] The culture medium was MS supplemented with 3% sucrose.

Germination of secondary somatic embryo (SSE)

Increasing in time period for maintaining SSEs on MS supplemented with 0.2 M sorbitol before transferred to MS medium devoid plant growth regulators yielded the increment of germination of SSE by producing shoot and root (Table 2). When time period were increased from 2 to 3 months on those medium without subculture promoted germination of shoot more than 20 times of one month culture. Complete plantlets regeneration (both shoot and root) formation at 3.7% was obtained from 3 months maintenance whereas 1 and 2 months failed to produce complete plantlets (Table 2). Cluster of torpedo-stage SSE were elongated and individually isolated which further develop into complete plantlets on hormone-free MS medium with 3% sucrose for 3 months (Fig. 4B and C). This time period (3 months) of maintenance on sorbitol containing medium was enough to stress those SSE to be ready for germination. This process might relate to the hydrolysis of food reserves in the endosperm and the mobilization of nutrients required for embryos germination like the report of Sarasan (2005). Those complete plantlet could be readily excised and transferred to soil. In case of embryos that germinated only shoots, it was necessary to induce root before transferring to soil.

Table 2. Effect of time period for maintenance SSEs on MS medium supplemented with 0.2 M sorbitol and 200 mg/l As on germination after culturing on hormone-free MS medium with 3% sucrose for 3 months.

Time period (months)	SSE producing shoot (%)	SSE producing root (%)	Complete plantlet (%)
1	0.74	4.44c [1/]	0.00b
2	15.55	12.59b	0.00b
3	31.10	29.30a	3.70a
F-test	ns	**	**
CV(%)	33.96	4.34	47.40

ns = Non significant. ** = Significant at P<0.01 level. [1/] = Value followed by different letter are significantly different according to DMRT-test at P<0.01 level.

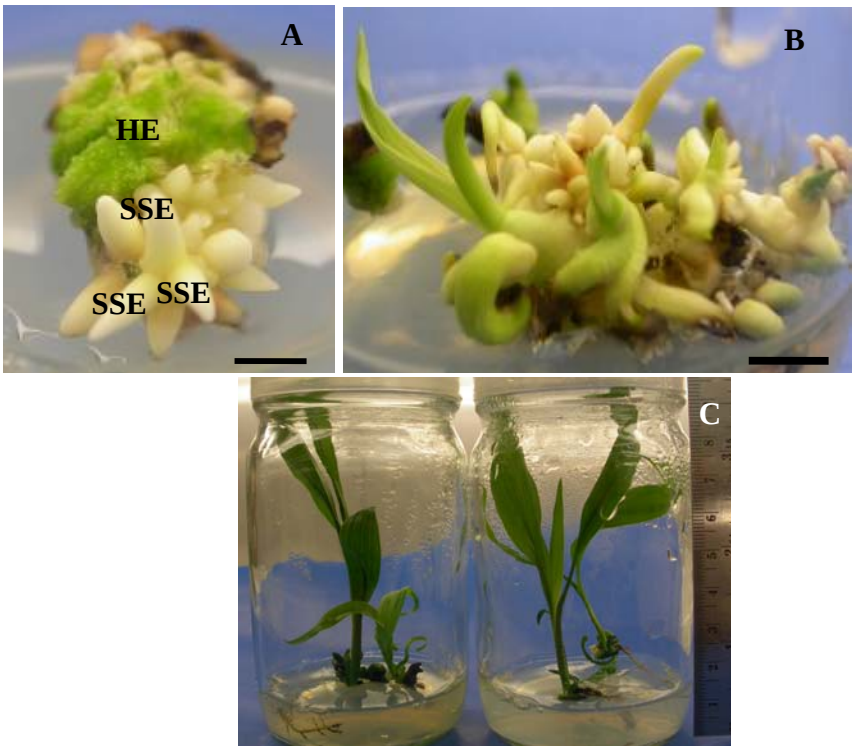


Fig. 4. Development of SSE from HE (A) on 0.2 M sorbitol containing MS medium for 1 month of culture (bar: 3.25 mm) subsequent to germination of SSE (B) on hormone-free MS medium (bar: 6 mm) for further 3 months. (C) Complete plantlet ready transfer to soil.

Conclusion

The present study successfully describes *in vitro* plant regeneration protocol of MZE via somatic embryogenesis. The highest percentage of HEs (14) was obtained from 865(D)×110(P) while the highest percentage of EC (26.03) was obtained in 174(D)×206(P). MS medium supplemented with 0.5 mg/l dicamba gave the best response on EC proliferation. SSEs were achieved at 80 % on MS medium supplemented with 0.2 M of sorbitol and 200 mg/l As. Plantlet conversion rate at 3.7 % from SSEs was obtained from 3 month maintenance of HE on sorbitol containing medium subsequent to culture on PGR-free MS medium.

Acknowledgment

The authors are grateful to the Director, Klong Hoi Khong Research Station. Financial assistance provided by Graduate School, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

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(Received 24 July 2008; accepted 23 October 2008)