AcademicPres

Available online at www.notulaebotanicae.ro

Print ISSN 0255-965X; Electronic 1842-4309

Not. Bot. Hort. Agrobot. Cluj 38 (1) 2010, 213-217

Notulae Botanicae Horti Agrobotanici Cluj-Napoca

The Effect of Chitosan on the Organogenesis of Oil Palm Embryo-Derived Callus

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Abstract

Zygotic embryos of oil palm (*Elaeis guineensis* Jacq. var. *tenera*) were excised and cultured on MS medium containing 3 mg/l 2, 4-D either with or without 0.05% activated charcoal (AC). Improved growth of embryos was obtained on MS medium supplemented with 0.05% AC. Callus cultures were initiated from embryos, young leaves and roots on an MS medium containing 2, 4-D, NAA and 0.05% AC. On these media, two morphologically distinct types of white and yellow compact calluses were produced. Green shoots regenerated after several transfers of the yellow compact calluses from zygotic embryos to MS medium supplemented with 15 mg/l chitosan either with or without 5 mg/l 2, 4-D. Histological sectioning revealed that regenerated shoots originated from a clump of meristematic cells that had dense cytoplasm. Regenerated shoots rooted when transferred to the MS medium in the presence of 0.05% AC. Transfer of plantlets to the soil was achieved. Callus from young seedling leaves and roots did not regenerate shoots or roots in a medium containing 2, 4-D or TDZ, with or without chitosan. This finding shows that chitosan can initiate organogenesis in oil palm callus.

Keywords: auxin, callus culture, chitosan, *Elaeis guineensis* Jacq., regeneration

Introduction

Among the commercially important oil yield plants, oil palm (*Elaeis guineensis* Jacq.) is a valuable economically important source of vegetable oil. It is an arborescent monocotyledon native of Africa and now widely cultivated across Southeast Asia. At present, palm oil is the most traded vegetable oil in the international market, and is increasingly used in the food industry. In the world's supply, it takes second place only after soy bean. Thailand is the world's fourth largest producer of palm oil and third largest exporter. Consequently, palm oil production represents a significant and important part of the Thai economy. However, there is much room for improvement because the current average yield of palm oil in Thailand is only 2.8 tons per hectare compared to 3.6 tons per hectare in Malaysia. Within a relative short period the area planted with oil palm has increased from 15,000 hectares in 1980 to 199,000 hectares in 2000, showing a growth rate of 7.8% per annum (Basiron, 2002).

Palm oil is generally propagated by seeds therefore a great variation in the plantation is expected due to the heterozygosity of the seedlings. In addition, seed germination of some cultivars such as *Psifera* (Shell-less; embryo rarely form) is very poor. Conventional vegetative propagation in this woody allogamous species is not possible and tissue culture has been used as a tool to overcome this impediment. Plant regeneration via *in vitro* culture has been initiated from various sources of explants (Teixeira *et al*., 1995; Patcharapisutsin and Kanchanapoom, 1996; Sambanthamurthi *et al*., 1996; Aberlenc-Bertossi *et al*., 1999). Embryo culture has proved useful in increasing the number of seedlings and it is interesting to use as a convenient source of explant for callus induction. Furthermore, embryo culture is an important prerequisite for the successful application of several *in vitro* techniques. Zygotic embryos of palms have been mainly used as explants for induction of callus with generative capacity as in coconut palm (Fisher and Tsai, 1978; Gupta *et al*., 1984), date palm (Gabr and Tisserat, 1985), Christmas palm (Srinivasan *et al*., 1985), sago palm (Alang and Krishnapillary, 1986), *Hyophorbe amaricaulis* palm (Douglas, 1987), *Washingtonia filifera* palm (DeMason, 1988), Canary Island date palm (Le Thi *et al*., 1999) and macaw palm (Moura *et al*., 2008). A callus-mediated plant regeneration protocol is a prerequisite and fundamental requirement for the exploitation and improvement of this plant through genetic transformation (Abdullah *et al*., 2005; Lee *et al*., 2006).

Although most of plant regeneration in oil palm was produced through callus and cell suspension cultures the growth of either calluses or cells in cultures is very slow. Therefore most culture media were modified by the addition of auxins or cytokinins to accelerate efficient regeneration. Information concerning details of media and growth regulator amendments is still a fundamental requirement of the intense commercial production. In recent years

chitosan has attracted notable interest due to its biological activities such as antimicrobial, antitumor and stimulant of plant growth. Chitosan is a natural carbohydrate polymer derived by deacetylation of chitin which consists of N-acetyl-D-glucosamine and D-glucosamine residues linked by β-1, 4 glycosidic bonds (Chibu and Shibayama, 2001). Chitosan is an exogenous elicitor and plays a role in plant resistance to pathogens and defense mechanism and has been shown to affect plant growth (Uthairatanakij *et al.*, 2007). Hence the current paper reports the effect of chitosan with the objective of developing a protocol for the regeneration of oil palm from the callus.

Materials and methods

Plant materials

Mature fruits of oil palm *Elaeis guineensis* Jacq. var. *tenera* were provided by a local oil palm company in Nakhon Si Thammarat Province, Thailand and dehusked manually. Seeds were removed from the fruits and used in the experiment. The exposed hard endocarp was cracked open with a hammer and the kernels (zygotic embryo embedded in endosperm) were surface sterilized by a 10-min immersion in 70% (v/v) ethanol and then cut into small cubes. These cubes with embedded zygotic embryos were then surface sterilized by a 30 min. immersion in 40% (v/v) Clorox® solution of commercial laundry bleach (5.25% NaOCl) containing 1-2 drops of Tween 20® per 100 ml of solution. Thereafter, the kernels were rinsed several times with autoclaved distilled water and kept for 24 hours prior to excision. Zygotic embryos were aseptically removed from the kernels with a scalpel and transferred to the culture medium.

Culture media

The excised embryos were initially inoculated on MS (Murashige and Skoog, 1962) basal medium supplemented with or without 0.05% AC in order to induce seedlings. The initial cultures and subsequent recultures of embryos were incubated for a period of 8 weeks. Callus production was initiated from zygotic embryos, young leaves and root derived from seedlings. The zygotic embryos were cultured on MS medium supplemented with either 0 or 3 mg/l 2, 4-D and either with or without 0.05% AC. Young leaves were cultured on MS medium supplemented with 0.05% AC and either with 2, 4-D at 0, 1.5, 2.0, or 2.5 mg/l or NAA at 0, 10, 20, or 30 mg/l. Young roots were cultured on MS medium supplemented with 0.05% AC and either with 2, 4-D at 0, 3, 5, or 7 mg/l or NAA at 0, 30, 50, or 70 mg/l. Callus initiation from embryos, young leaves and roots and the percentage of explants forming callus was recorded after 8 weeks of culture. Calluses were subcultured every 2 weeks on these media for 4 months. The resulting calluses were transferred to the MS medium supplemented with 0.05% AC and one of the following growth regulators: 2, 4-D (5 mg/l), TDZ (1, 3, or 5 mg/l), chitosan (10, 15, or 20 mg/l). Plant regeneration was recorded at two week intervals for a period of 4 months.

Culture conditions

All media were solidified with 0.15% Gelrite. The concentration of sucrose was 3% (w/v) and the pH of all media was adjusted to 5.6 with 0.1 N NaOH or HCl prior to the addition of Gelrite. The media were autoclaved at 121°C for 20 min and 25-ml aliquots were dispensed into 115-ml screw-topped jars. Cultures were incubated at 26 ± 1°C with a 16 h photoperiod under an illumination of 20 μmol $m^{-2}s^{-1}$ photosynthetic photon flux density provided by Gro-Lux light.

Plant acclimatization

Regenerated shoots were transferred to the basal MS medium supplemented with 0.05% AC to promote root elongation for 3 weeks. Gelrite was carefully washed from the well-rooted plantlets and they were then transferred to black plastic pots filled with potting soil covered with transparent plastic sheet and watered with MS medium lacking sucrose: distilled water 3: 1 for a period of 2 weeks under greenhouse conditions. Then transparent plastic sheet was taken off and plantlets were left to harden for an additional 4 weeks under shading, natural photoperiod and high relative humidity.

Histological observations

For histological studies, calluses at different stages of development were fixed in FAA II solution of 90 ml 70% ethyl alcohol, 5 ml glacial acetic acid and 5 ml formalin solution. These calluses were dehydrated through an ethanol-tertiary butanol series for 48 h and embedded in Paraplast. Specimens were sectioned at 10 to 14 μm and stained with safranin and fast green (Sass, 1958). All sections were mounted with Permount and were viewed under bright-field illumination with an Olympus microscope. Histological analysis was carried out on representative samples of the callus explants.

Statistical analysis

One explant was planted per jar and all experiments were carried out with 15 replicates per treatment and the experiments were repeated on three consecutive days. The percentage of callus induction and number of regenerated shoots were monitored as growth parameters. Data on callus formation were submitted to analyses of variance and the different responses of explants to various plant growth regulators were compared using Sheffe's test at P≤0.05. The software used was Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA) for Windows XP Professional.

Results and discussion

Tab. 1. Effects of 2, 4-D and activated charcoal on growth of oil palm embryos and callus induction cultured on MS medium for 8 weeks

Combination number	2,4-D (mg/l)	Activated charcoal (%)	Germination (%)	Callus formation (%)
1	0	0	65b	0c
2	0	0.05	85a	0c
3	3	0	0c	67b
4	3	0.05	0c	82a

Each value represents the mean of fifteen replicates means within a column followed by different letters show significant difference as analyzed by Sheffe's tes at p≤0.05

Tab. 2. Effects of 2, 4-D and NAA on callus induction of oil palm leaves and roots cultured on MS medium containing 0.05% AC for 8 weeks

Combination number	Plant growth regulators (mg/l) 2,4-D	NAA	Callus formation (%)	Explant mortality (%)
Leaf explant				
1	0	0	0c	100a
2	1.5	0	0c	15e
3	2.0	0	15b	30d
4	2.5	0	20a	40c
5	0	10	0c	10e
6	0	20	0c	40c
7	0	30	0c	80b
Root explant				
8	0	0	0d	10a
9	0	30	44b	0b
10	0	50	65a	0b
11	0	70	25c	0b
12	3	0	0d	0b
13	5	0	0d	0b
14	7	0	0d	13a

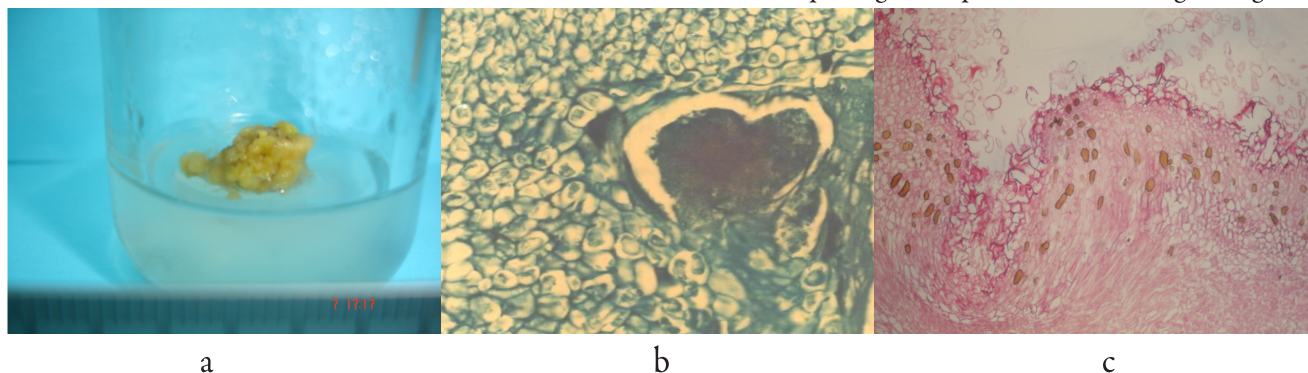
Each value represents the mean of forty five replicatesmeans within a column followed by different letters show significant difference as analyzed by Sheffe's test at p≤0.05

During the first week incubation, all embryos slightly swelled. Germination normally occurred during the first 4-8 weeks on medium without auxin but some embryos did not germinate at all. After two months, the highest germination (85%) and growth of both roots and shoots was recorded on MS basal medium with or without 0.05% AC (Tab. 1). This is probably because AC prevents explant browning upon wounding of the cultured embryos. The effect of AC is attributed to adsorption metabolites such as polyphenols and quinones released by the plant tissues (Wang and Huang, 1976). Seedlings usually showed normal geotropic responses and no callus production was observed.

Embryos gave rise to compact, yellow creamy callus eight weeks after culture initiation on MS medium containing 3 mg/l 2, 4-D with or without 0.05% AC (Tab. 1, Fig. 1a). Production of regenerative callus with generative capacity from excised zygotic embryos was observed in several palm species (Fisher and Tsai, 1978; Gupta *et al*., 1984; Gabr and Tisserat, 1985; Srinivasan *et al*., 1985; Alang and Krishnapillary, 1986; Le Thi *et al*., 1999; Moura *et al*., 2008). Callus production on explants has been observed on MS medium supplemented with 2, 4-D and 0.05% AC with the highest percentage of callus formation being in 2.5 mg/l 2, 4-D (Tab. 2).

It seems clear that 2, 4-D, the most commonly used auxin for embryoid induction is required for callus initiation. The initial callus was yellow in color and friable in appearance and grew slowly unlike those initiated from embryos. No callus formation was observed in the presence of NAA (Tab. 2). For callus induction in roots, the texture of callus was white and friable and the highest callus formation was 65% in 50 mg/l NAA and no callus was produced at none of the 2, 4-D concentrations (Tab. 2).

Attempts were made to initiate plantlet production through the application of 2, 4-D, TDZ and chitosan. All calluses obtained from zygotic embryos, young leaves and roots were transferred to these media. Only yellow compact calluses obtained from zygotic embryos cultured on MS medium with 3 mg/l 2, 4-D and 0.05 % AC showed distinct morphological responses and the fast growing cal-



a b c

Fig. 1. Formation and histological study of oil palm callus. (a)Yellow compact callus derived from embryo formed on MS medium containing 3 mg/l 2, 4-D. (b) A group of meristematic cells with densely stained cytoplasm and nucleus. (c) Cells containing high storage lipid content as droplets. Bar = 100 µm

Tab. 3. *In vitro* responses of granular callus of oil palm cultured on MS medium supplemented with 2, 4-D, TDZ and Chitosan

Combination number	Plant growth regulators (mg/l)			Shoot formation (%)	Root formation (%)
	2, 4-D	TDZ	Chitosan		
1	0	0	0	0c	0e
2	0	1	0	0c	0e
3	0	3	0	0c	0e
4	0	5	0	0c	0e
5	0	0	10	0c	0e
6	0	0	15	16a	0e
7	0	0	20	3c	0e
8	5	1	0	0c	75a
9	5	3	0	0c	60b
10	5	5	0	0c	50c
11	5	0	10	0c	50c
12	5	0	15	12b	35d
13	5	0	20	2c	30d

Each value represents the mean of forty five replicates means within a column followed by different letters show significant difference as analyzed by Sheffe's test at $p \leq 0.05$

luses were obtained. The newly formed callus was granular and granules could be separated from each other easily. The acquisition of organogenic competence was influenced by the presence of chitosan (Tab. 3). A histological study showed that callus produced clumps of cells with a markedly meristematic appearance (Fig. 1 b).These cells were small with dense cytoplasm and contained a well-stained nucleus. Continuity of division led to intense proliferation and differentiated cells containing numerous storage lipids which were clearly visible as droplets within the cytoplasm (Fig. 1 c).

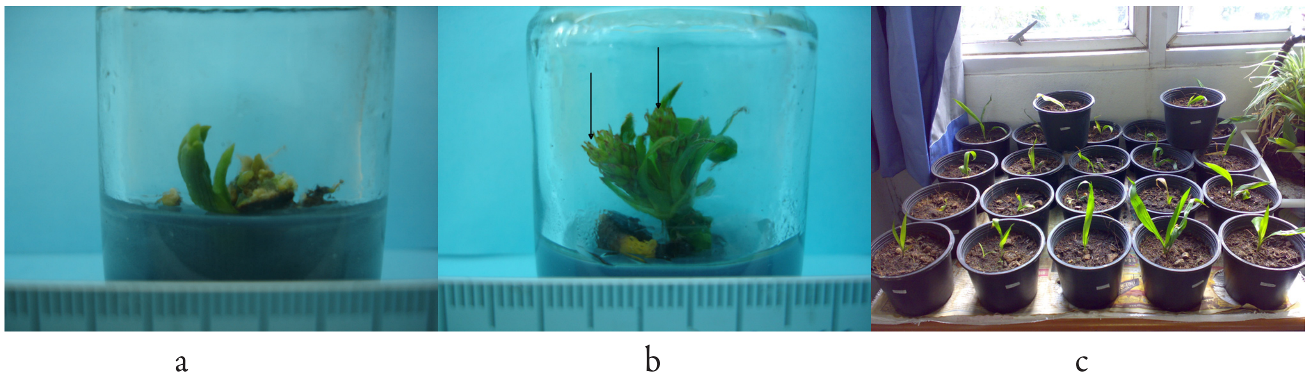
Shoot-like growth without roots emerged on MS medium containing 15 mg/l chitosan either with or without 5 mg/l 2, 4-D (Fig. 2 a). Chitosan is a major component of the shells of crustaceans and the second most abundant natural biopolymer after cellulose. Chitosan is now widely produced commercially and has been documented to stimulate plant growth (No *et al.*, 2007). A positive effect of chitosan was observed on the growth and development of *Dendrobium phalaenopsis* orchid both in liquid and on solid medium (Nge *et al.*, 2006). In the present study, the exogenous application of chitosan induced shoot organogenesis from the embryo derived callus suggesting that the role of chitosan might provide the stimulant effect. In addition TDZ, a substituted phenyl urea, had a tendency to turn callus to a non-embryonic state and this result is in agreement with Rajesh *et al.* (2003).

It is interesting to note that prolonged culture on chitosan containing medium produced in vitro flower (Fig. 2 b). Corley *et al.* (1986) showed that the incidence of mantled flower (the flowers are non-functional and there is no fruit development) in oil palm culture increases with time cultures that have been maintained at the embryoid multiplication stage. Prolonged culture on media containing high levels of plant hormones may disrupt the normal hormone metabolism of the cells in some cases. In this study, flower malformation is probably due to time in culture or chitosan application.

For root induction, each shoot was excised and transferred to MS medium containing 15 mg/l chitosan in the presence of 0.05% AC for 3 weeks and rooting was achieved on this medium. All plantlets with well developed shoot and root were acclimatized and they subsequently continued to grow into fairly uniform plants (Fig. 2 c). However, the percentage of shoot formation has to be improved since only 16% was obtained (Tab. 3).

Conclusions

In conclusion plant regeneration through organogenesis from oil palm embryo derived callus is described. These calluses showed a better response to chitosan giving rise to shoot organogenesis at 15 mg/l chitosan either with or without 5 mg/l 2, 4-D. This interesting property of chitosan suggests its potential for the micropropagation of oil palm. Hence a better understanding in elucidating the



a b c

Fig. 2. Organogenesis in oil palm callus. (a) Regeneration of plant derived from yellow compact callus cultured on MS medium containing 15 mg/l chitosan. (b) Development of *in vitro* inflorescence. (c) Six-month-old plantlets obtained from organogenesis in potting soil

mechanism of chitosan could improve the efficiency of organogenesis in this oil bearing plant.

Acknowledgements

This work was partially funded by the Royal Thai government annual budget through the Graduate School, Prince of Songkla University, Hat Yai, Songkhla 90112.

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