Endosperm Culture of *Jatropha curcas* L.

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ABSTRACT

Endosperm culture of Jatropha curcas L. or physic nut is an interesting method, but no previous studies have been reported. Endosperm explants were cultured on Murashige and Skoog media supplemented with 30 g/L sucrose and various concentrations of 2,4–dichlorophenoxyacetic acid (2,4–D), 1ñnaphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA) i.e. 0, 5 and 10 µM, and kept under darkness for 45 days. The results showed that the highest percentage of callus induction was found on the medium supplemented with 10 µM NAA (100%) followed by 10 µM 2,4–D (92%) and 10 µM IBA (86%). However, no callus was observed in the control medium and medium supplemented with 5 µM 2,4–D, NAA and IBA. In addition, the largest compact yellow callus was found on the medium with 10 µM 2,4–D and NAA while small yellowish callus was found on the medium with 10 µM IBA. In further study, friable callus derived from endosperm were used for suspension culture. Growth rate of suspended cells and percentage of oil content during growth period were measured. The results revealed that endosperm cells could grow and could produce oil. The suspended cells grow rapidly during 10–15 days of culture and gave the maximum percentage of oil content 16.08% (w/v) at 15 days of culture then decreased to 4% on the last day of culture (40 days).

Key words: *Jatropha curcas* L., Callus induction, Endosperm culture

INTRODUCTION

Jatropha curcas L. or physic nut, is a shrub plant found in tropical area. In recent years, this plant has received an extensive attention as an energy plant, because of its seed oil content. However, it still has some limitations, i.e. difficult harvest and low yield.

Polyploids are reported to have some advantage over diploids. In case of oil crops, they show better grain filling, high oil content, enhanced photosynthetic ability, delayed maturity and increased biomass (Li et al., 1999). Generally, triploids are raised by crossing tetraploids with a diploid parents. Endosperm culture is an alternate way to produce the triploid plant. The objectives of this studies were to find out a formulation of medium that could be used to induce callus from the endosperm and to study the possibility to produce oil from endosperm suspended cells *in vitro*.

MATERIALS AND METHODS

Plant materials

Mature seeds of physic nut were harvested from Mae Hia Agricultural Research and Training Centre, Faculty of Agriculture, Chiang Mai University, in August 2009.

Seeds sterilization and initiation

Mature seeds of physic nut were washed under running tap water about 15 minutes to remove soils and other contaminants on the outer surface of seed coats. After that, the seeds were thoroughly rinsed in 70% (v/v) ethanol for 1 minute and then were washed three times in sterilized distilled water. Additionally, the seeds were surface sterilized with mercuric chloride ($HgCl_2$) 0.1% (w/v)

for 15 minutes and they were washed three times in sterilized distilled water. After that, the seeds were immersed in 95% ethanol and they were briefly flamed. Endosperm from seeds were cut to size of 0.5x0.5 cm.

Media and culture conditions

Medium used to induce callus from these explants was Murashige and Skoog (MS) medium (1962), 3% (w/v) of sucrose and 0.8% (w/v) agar were added. The pH was adjusted to 5.6±0.02 using a pH meter prior to autoclave at 121°C for 15 min. All culture incubated at 25±1°C, in darkness condition.

Callus induction

Callus induction studies were carried out by culturing the steriled endosperm explants in Murashige and Skoog (MS) media containing 2,4–D, NAA and IBA at concentrations of 5 and 10 μM. The control medium was MS without any addition of plant growth regulators. A total of thirty explants were cultured in each treatment with three replications. The percentage of explants forming callus, degree of callogenesis and morphologies (callus texture, callus color) were observed.

Cell suspension culture

Cell suspension cultures were initiated by inoculating 1 g of fresh friable callus into 30 ml of MS liquid media. Medium for suspension culture was supplemented with 10 μM/L NAA. Cultures were incubated continuously on a rotary shaker (120 rpm) under dark condition at 25±2°C for 40 days.

Growth measurement and analysis of oil content

Number of cells, packed cell volume (PCV), fresh weight (FW) and dry weight (DW) of cells were measured as growth. Oil production of suspended cells was measured and expressed in terms of total lipids extract (TLE). Total lipid extract analysis was conducted using the method from Zou et al. (1995). The cells were mixed with 1 ml isopropanol, followed by sonication for 10 minutes. The mixtures were then heated in boiling water for 5 minutes. The solution was immediately cooled, prior to addition of 0.5 ml of CH_2Cl_2. The mixture was set aside for 30 minutes at room temperature, and shaked occasionally using a vortex mixer. The organic and aqueous phases were then separated by the sequential addition of 2 ml of CH_2Cl_2 and of 2 ml of 1 M KCl in 0.2 M H_3P0_4. After the sample was centrifuged (x500g) for 5 minutes, the lower organic phase was collected and the aqueous phase was washed twice with 2 ml of CH_2Cl_2. The original organic phase was then mixed with the washes and dried to yield the TLE. The growth of suspended cell and TLE analysis were measured every 5 days until 40 days. All parameters were repeated three times.

Statistical analysis

Each experiment was set up under a completely randomized design (CRD). Data were subjected to one–way ANOVA. Differences among means were tested for significance by Duncan's new multiple range test at 0.01 level of probability.

RESULTS AND DISCUSSION

Callus formation

Endosperm explants of physic nut could not grow in the control medium which lacked plant growth regulators (Figure 1A). The explants cultured in control medium turn brownish in colors and died after one month of culture. Consistent with other research, for example Thomas et al. (2000) who observed that callus did not formed in immature endosperm. For the callogenesis from the endosperm explants of physic nut, calli were initiated from the cut margins of the explants in the presence of suitable plant growth regulators (Figure 1B).

Three types of plant growth regulators were tested; 2,4–D, NAA and IBA. It was found that the concentration at 10 µM NAA was the best for callus induction (100%), followed by 10 µM 2,4–D (92%) and 10 µM IBA (86%) respectively. However, at the concentration 5 µM, 2,4–D, NAA and IBA, the explants could not induce callus formation. These results showed that types of auxin could induce callus formation of the endosperm. However, only the appropriate concentrations of auxin can induced callus formation (Table 1).

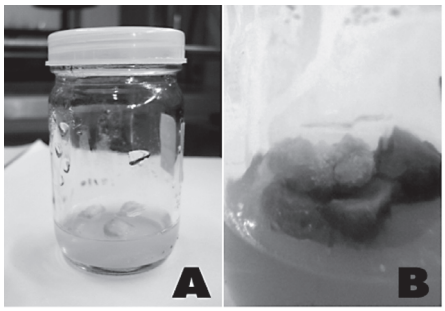

Figure 1. Callus induction from physic nut endosperm. A. No callogenesis in hormone free MS medium. B. Callogenesis at the cut margin of the explants in the presence of suitable medium supplemented with appropriate type and concentration of auxin.

The degree of callus formation, type of callus or callus texture and color of callus, were found that medium supplemented with 10 µM 2,4–D and NAA gave average callus (1.0–2.0 cm). They were compact yellow (++) and pale yellow (+) respectively. Medium supplemented with 10 µM IBA gave smaller callus (less than 1.0 cm). They were friable pale yellow (+) (Figure 2A–2F).

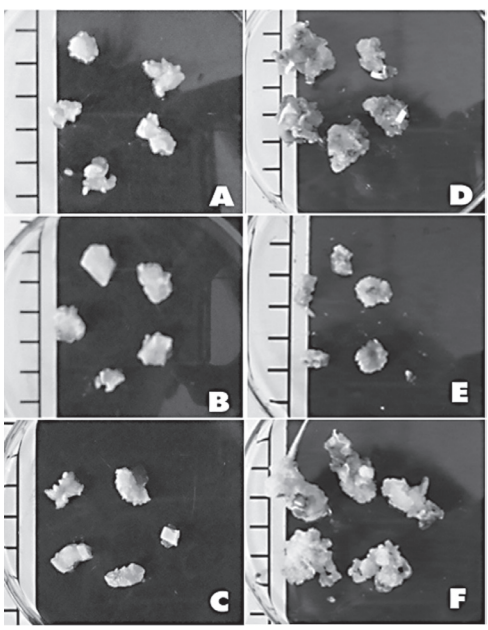

Figure 2. Callogenesis of physic nut endosperm in MS medium supplement with various auxins at different concentrations. A. 5 µM 2,4–D; B. 5 µM IBA; C. 5 µM NAA; D. 10 µM 2,4–D; E. 10 µM IBA; F. 10 µM NAA.

Table 1. Effects of 2,4-D, NAA and IBA in MS media on callus induction of physic nut endosperm explants.

Plant growth regulator	Concentration (μM)	Percentage of callus formation (%)[1]	Degree of callus formation[a]	Type of callus[b]	Color of callus[c]
Control	0	0 c	-	-	-
2,4-D	5	0 c	-	-	-
	10	92 ab	Average	C	Y (++)
NAA	5	0 c	-	-	-
	10	100 a	Average	C	Y (+)
IBA	5	0 c	-	-	-
	10	86 b	Poor	F	Y (+)

[1]Means followed by the same letters are not significantly different at $P = 0.01$ by Duncan's new multiple range test.
[a]Degree of callus formation: absence of callus (-), less than 1.0 cm (poor), 1.0–2.0 cm (average), more than 2.0 (good)
[b]Type of or callus texture: compact callus (C), friable callus (F)
[c]Color of callus: absence of callus (-), white (W), pale yellow (Y (+)), yellow (Y (++)), dark yellow (Y (+++)), brown (B)

Growth measurement and analysis of oil content

The friable calli were cultured on MS solid media (Figure 3A). Cell suspension cultures were established by using 30 day-old calli as inoculums. One gram of actively growing friable calli were transferred to MS liquid medium supplemented with NAA 10 μM/L. After 7 days of inoculation, the cells were filtered through a sieve (40 mesh or 380 μm) and these cells were used for subculture (Figure 3B). Thereafter, the growth rate and the oil production were measured every 5 days until 40 days of culture. It was found that friable calli of physic nut were easily broken apart and dispersed into single cells or aggregated cells (Figure 4A, 4B). Under the light microscopy, cells had many different shapes (Figure 4C), some cells were elongated and highly vacuolated with sparse cytoplasm (Figure 4D).

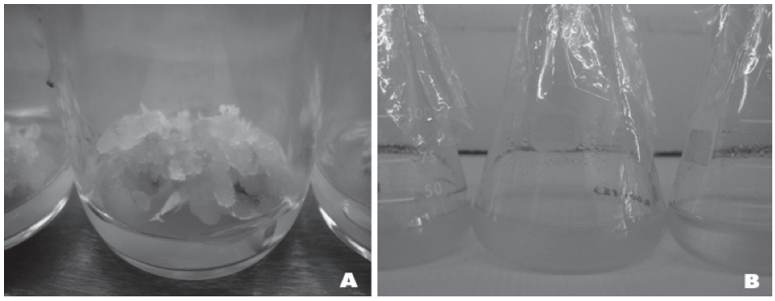

Figure 3. Cell suspension culture of physic nut endosperm. A. Friable callus in MS - N10 solid media. B. Suspended cells in liquid media after filtrated.

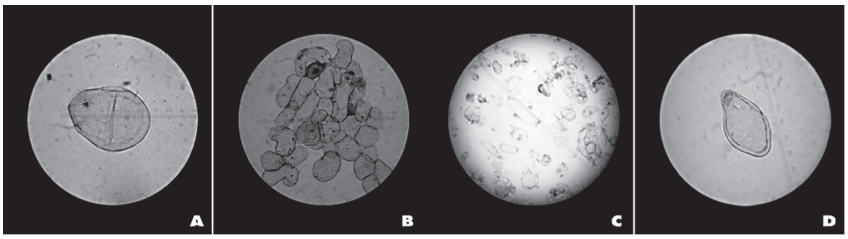

Figure 4. Different of suspended cells in suspension culture of *Jatropha curcas* L. A. Single cell, B. Aggregated cells, C. Different of cell shapes in suspension culture, D. Highly vacuolated in some cells.

The growth of suspended cell was shown in Figure 5A–5C. The results indicated that the growth rate of suspended cells was initially slow during 5 days (lag phase). The cell had the maximum growth; number of cells, packed cell volume (PCV), fresh weight (FW) and dry weight (DW), at 15th day (log phase). The growth was slowly down to be stable at 20th day (stationary phase).

The total lipid extracts (TLE) were shown in Figure 5E. The results indicated that the suspended cells produced oil. The oil content of suspended cells were initially slow during 5 days about 5% (w/v) and increased to highest 16.08% (w/v) at 15 days of cell suspension cultures. The result was agreed with Hapsari et al. (2011) who reported that calli from hypocotyls explants from physic nut had the TLE only 15.2% (w/v) and TLE content increased along with maturation of culture stage. In this study, the oil contents were rapidly decreased after culture for 15 days to the 40 days. On the last cultured day, the oil contents were about 4% (w/v).

From these results it was shown that the suspended cells (endosperm cells) can be grown and produced oil in the liquid MS medium supplemented with 10 µM/L NAA alone. The growth and the oil content tend to be change in the same pattern. It indicated that the improvement in the growth of suspended cells might affect oil content. The exogenous factors i.e., temperature, osmotic potential and externally applied plant hormones have an effects the storage lipids including; triacylglycerol (TAG) content and fatty acid composition in various plant species (Kharenko et al., 2010).

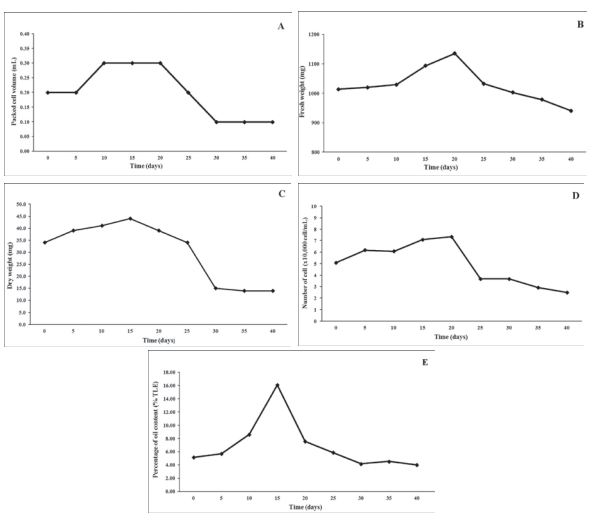

Figure 5. Growth rate of cell suspension culture of physic nut endosperm. The data represent the mean values from three individual flasks of cell suspension culture ± SD. A. Packed cell volume (PCV); B. Fresh weight; C. Dry weight; D. Number of cell (x10,000 cell); E. Percentage of oil content (% TLE).

CONCLUSION

In conclusion, it was found that media supplement with 10 µM NAA was the most efficient formulation for callus induction (100 %), which followed by media supplemented with 10 µM 2,4–D and 10 µM IBA, shown percent of callus formation 92% and 86%, respectively. However, callus was not formed in the control media and media supplemented with 5 µM 2,4–D, NAA and IBA. The media supplemented with 10 µM 2,4–D and NAA gave average callus (1.0–2.0 cm). They were compact yellow (++) and compact pale yellow (+), respectively. While media supplemented with 10 µM IBA was giving smaller callus (less than 1.0 cm). It was friable pale yellow (+). In addition, the friable calli of physic nut derived from endosperm were used for a suspension culture. The growth rate of suspended cells and the percentage of oil content during growth period were measured. The results revealed that endosperm cells could grow and produce oil. The suspended cells grew rapidly during 10–15 days of culture and gave the maximum percentage of oil content 16.08% (w/v) at 15 days of culture and decreased to 4% on the last day of culture (40 days).

In the further study, we will find the optimum condition for the growth and the accumulation of oil content, such as temperature, type and concentration of carbon source and exogenously phytohormone. This study is the first report about cell suspension culture obtaining from endosperm explants and the production of oil content from suspended endosperm cells of physic nut.

ACKNOWLEDGEMENTS

The authors would like to express the sincere thanks to Office of the Higher Education Commission (OHEC), Thailand for supporting the research fund. We also would like to say thanks to Associate Professor Dr. Pornchai Lueang-a-papong for accessing to Jatropha trees at Mae Hia Agricultural Research and Training Centre, Faculty of Agriculture, Chiang Mai University.

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