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IN DEPTH STUDY FOR DEVELOPING EFFICIENT DIRECT SOMATIC EMBRYOGENESIS OF *JATROPHA CURCAS* L.: MORPHOLOGY AND ULTRASTRUCTURE

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Abstract

This is the first report on direct somatic embryogenesis of *Jatropha curcas* L. For in depth study, morphological observation was performed by inverted microscopy whereas ultrastructural observation was conducted by transmission electron microscopy. The somatic embryos derived from single cells, which divided, developed into embryo proper with suspensor, ECS (Embryogenic cell suspension), proembryo, globular embryo, heart and torpedo stage embryo.The shoot and plantlet developed when the ECS was plated on embryo induction medium I, embryo induction medium II and embryo germination medium, consecutively. The highest germination percentage was 63.3% on IG_4-gamborg medium supplemented with 3 ppm GA3 and 1 ppm IAA. The highest shoot development was on IG_4-gamborg (60%) and the highest plantlet development was on IG_4-glutamin (6.7%). The reason of low percentage of plantlet development was revealed by observing the ultrastructural features of the embryogenic cells and the somatic embryos. Character of the embryogenic cells and somatic embryo was forming deposit material in the vacuole. The deposit material inhibited cellular metabolism of the cells.

Introduction and Objectives

Current day awareness of the depletion of traditional energy resources has evoked an intense interest in alternative sources of energy. This has influenced trends in plant biotechnology, resulting in attempts to use cell and plant tissue for improvement and rapid propagation of plants producing oil with fuel application possibility. *Jatropha curcas* (L.) is one plant that has oil capable to substitute engine oil (biodiesel) (Nigam *et al.*, 2005).

The genus *Jatropha* belongs to Euphorbiaceae is one of the promising drought tolerant perennial species. The plant is very hardy and can tolerate high temperature and salinity. The seeds

contain semi-drying oil, an efficient substitutes for diesel engines. (Bhasubutra and Sutiponpeibun, 1982; Raina, 1987). Hundred thousand hectares of land in Nusa Tenggara Barat and Nusa Tenggara Timur, Indonesia will be planted by *Jatropha curcas*. Therefore, rapid and mass propagation of *Jatropha curcas* using somatic embryogenesis method is needed.

Sardana *et al.* (2000) carried out plant regeneration from somatic embryo of *Jatropha curcas*. They used solid media for somatic embryogenesis. They induced proembryos from green, compact, and slow growing embryogenic callus although their frequency was rather limited. Liquid medium has several advantages such as save place, labour, time, cost of medium and material. In 20 ml of liquid medium, Mariani *et al.* (2004) yielded ± 100 globular somatic embryo of *Lithospermum erythrorhizon*. Therefore, in 1 liter of liquid medium, we yielded ± 5000 globular somatic embryos. That result shows that much more somatic embryos were produced in liquid medium than in solid medium. In addition, the quality of somatic embryo in liquid media was also improved rather than in solid media. (Gupta et al., 2003). Accordingly, in this present study we reported direct somatic embryogenesis from single cell of *Jatropha curcas* in liquid media. Moreover, for in depth study, we reported observation of the somatic embryos of *J. curcas* by transmission electron microscopy.

Experimental

1. Optimization of embryogenic callus induction in *Jatropha curcas*

Seeds were germinated on moistened tissue paper. Hypocotyls from the germinating seeds were used as explant for somatic embryogenesis. The hypocotyl was cut and placed onto the Embryogenic Callus Induction Medium (ECIM) to induce friable embryogenic callus formation. ECIM consisted of Murashige & Skoog Basal medium supplemented with combination of 2,4-D (9 x 10^{-6} M, 1.35 x 10^{-5} M, 1,8 x 10^{-5} M) and BAP (0 M, 4,4 x 10^{-6} M), 2,4-D (9 x 10^{-6} M, 1.35 x 10^{-5} M, 1,8 x 10^{-5} M) and Kinetin (0 M, 4,4 x 10^{-6} M), IAA (1,14 x 10^{-5} M, 1.71 x 10^{-5} M, 2,28 x 10^{-5} M) and BAP (0 M, 5,7 x 10^{-6} M).

2. Cells suspension

The embryogenic callus was transferred to the cell suspension medium (CSM) to induce cells formation. The combination of 2,4-D and kinetin concentration was based on the best embryogenic callus induction medium. The best embryogenic callus induction medium was 1,35 x 10^{-5} M 2,4-D and 4.4.10^{-6} M kinetin. Therefore, the composition medium for CSM was 6,75 x 10^{-6} M 2,4-D and 4,4 x 10^{-7} M Kinetin.

3. Embryogenic cell suspension

The cell suspension in CSM was transferred to embryogenic cell suspension medium (ECSM). The composition of ECSM was MS medium supplemented with 1,35 x 10^{-5} M 2,4-D and 4,4 x 10^{-7} M Kinetin. In this medium KNO_3 was changed by 6 gL^{-1} K_3citrat.

4. Plantlet development

The embryogenic cells was subcultured to embryo induction medium I (EDMI) and embryo induction medium II (EDM II) before transferred to embryo germination medium (EGM). The composition of EDM I was half strength macronutrient of MS, micronutrient of MS, 10 ppm ascorbic acid, 20 ppm citric acid, 25 ppm adenine sulfat, 100 pm glutamine, 2 ppm kinetin and 1,5 ppm IBA.The composition of EDM II was the same as EDM I. supplemented with 3 ppm IAA and 3 ppm BAP. The composition of EGM was listed in Tabel 1.

Table 1. Plant growth regulator variation in embryo germination medium

GA_3 (ppm) / IAA (ppm)	0	Gamborg		Glutamin	
		2	3	2	3
0	control				
1		IG_3-gamborg	IG_4-gamborg	IG_3-glutamin	IG_4-glutamin

IG-glutamin medium consisted of ½ MS macronutrient+ MS micronutrient+ 10 ppm ascorbic acid+20 ppm citric acid+25 ppm adenine sulphate+ 100 ppm glutamine whereas IG-gamborg consisted of MS medium + Gamborg vitamin. Five time repletion was performed on each medium variation.

5. Transmission electron microscopy

Cells, embryogenic cells and the somatic embryos were prefixed in 0.1 M cacodylate buffer pH 7.2 containing 5% glutaraldehyde for 24 h, rinsed 3 times in the same buffer, postfixed in 2% osmium tetroxide in cacodylate buffer for 12 h, rinsed in the same buffer once and distilled water twice, and gradually dehydrated in an alcohol series, all at 4 degree C. The samples then were infiltrated and embedded with Spurr's resin at room temperature. The embedded samples were polymerized in an oven at 70 degree C for 24 h. Ultrathin sections were made with an ultramicrotome at a thickness of 70-90 nm. These sections were stained using aquaeous 2% uranyl acetate for 30 min, and with lead citrate for 10 min. Then, they were observed by TEM.

Results and Discussion

1. Optimization of embryogenic callus induction in *Jatropha curcas*

I . Basal medium JC with the addition of 2,4-D and BAP combination

2,4-D (M) / BAP (M)	9×10^{-6}	1.35×10^{-5}	$1,8 \times 10^{-5}$

0	Callus(+),White Yellow	Callus(+),White Yellow	Callus(+),White Yellow
4,4 x 10^{-6}	Callus(++),White Yellow	Callus(++),White Yellow	Callus(++),White Yellow

II. Basal medium JC with the addition of 2,4-D and Kinetin combination

2,4-D (M) / Kin (M)	9 x 10^{-6}	1.35 x 10^{-5}	1,8 x 10^{-5}
0	Callus (+), White Yellow, Friable (+), Wet	Callus (+), White Yellow, Friable (+), Wet	Callus (+), White Yellow, Friable (+), Wet
4,4 x 10^{-6}	Callus (++), White Yellow, Friable (++), Wet	Callus (++), White Yellow, Friable (+++), Wet	Callus (++), White Yellow, Friable (++), Wet

III. Basal medium JC with the addition of IAA and BAP combination

IAA (M) / BAP (M)	1,14 x 10^{-5}	1.71 x 10^{-5}	2,28 x 10^{-5}
0	Callus(+), White Yellow, Dry, Root(+)	Callus(+), White Yellow, Dry, Root(+)	Callus(+), White Yellow, Dry, Root(+)
5,7 x 10^{-6}	Callus(+), White Yellow, Dry, Root(+)	Callus(+), White Yellow, Dry ,Root(+)	Callus(+), White Yellow, Dry ,Root(+)

Note:

+ : a few

++ : moderate

+++ : abundant

Conclusion: The best embryogenic callus medium was basal medium JC supplemented with 1,35 x 10^{-5} M 2,4-D and 4.4.10^{-6} M kinetin.

Figure 1. Friable embryogenic callus of *Jatropha curcas* L.

Hypocotyl explant cultured on callus induction medium supplemented with combination of 2,4-D and BAP produced compact white yellow non embryogenic callus. This kind of callus was not suitable for cell suspension initiation. Hypocotyl explant cultured on callus induction medium supplemented with combination of IAA and BAP produced rooted dry white yellow callus. This kind of callus was also not suitable for cell suspension establishment. White yellow, friable and wet embryogenic callus was induced from hypocotyl explant on callus induction medium supplemented with combination of 2,4-D and kinetin. The best embryogenic callus was induced on callus induction medium supplemented with 1.35. 10^{-5} M 2,4-D and 4.4.10^{-6} M kinetin.

Cells in nodules at the surface of embryogenic callus was undegoing high proliferation cell and formed meristematic cell agregate, which have the potency became embryogenic cells in the suitable medium. (Filho and Hattori, 1997). Touchet *et al* (1991) found that nodular embryogenic callus consisted of single embryogenic cells. The aggregates were composed of typical meristematic cells containing soluble proteins and sometimes starch granules. They had a round prominent nucleus, and a dense cytoplasm with small vacuoles. These embryogenic masses were dividing actively. To check the embryogenicity of the callus, it was inoculated into cell suspension medium.

2. Somatic embryogenesis and embryogenic cell suspension

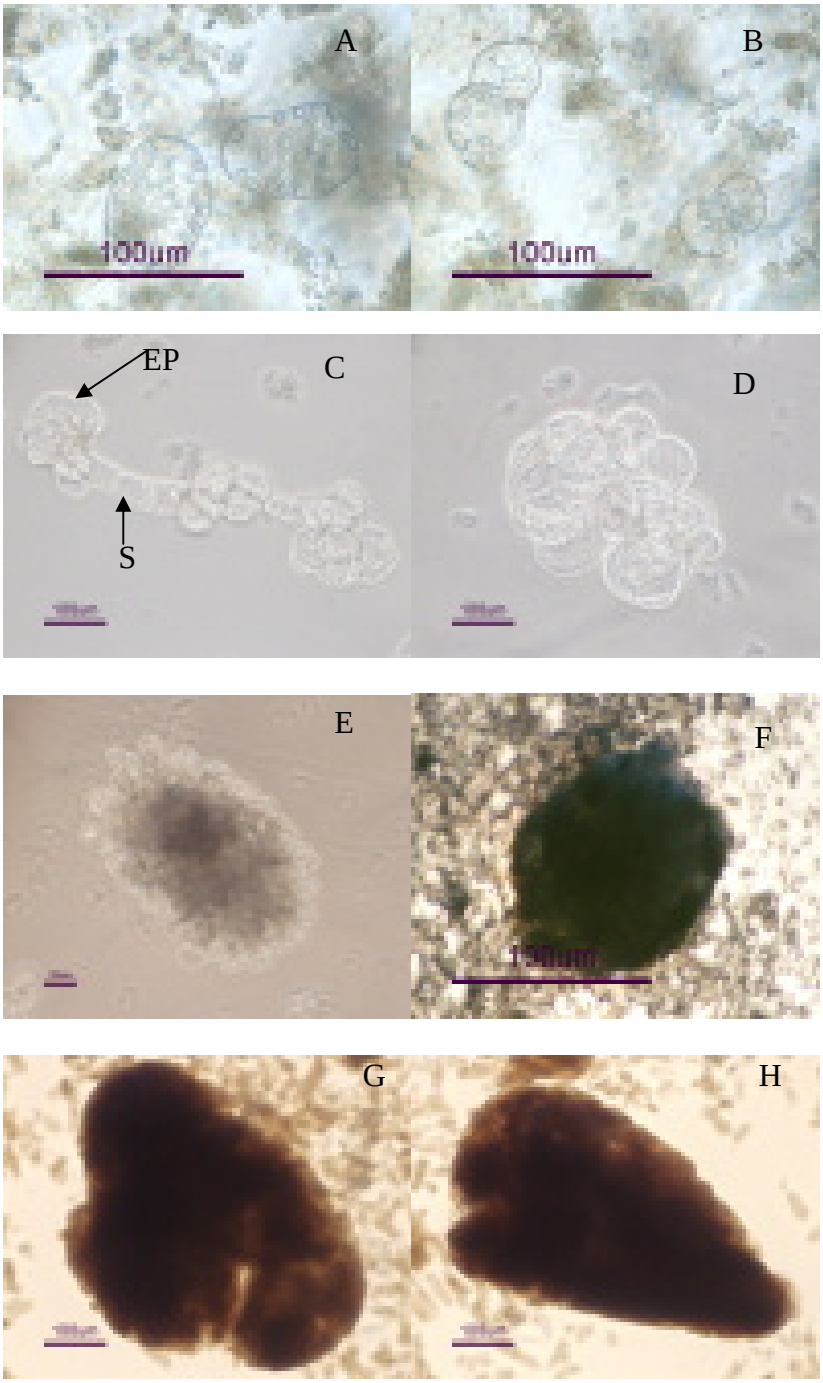

Figure 2 A-H. Single cells (A); Dividing cells (B); Embryo proper with suspensor (C); ECS (D); Proembryo (E); Globular somatic embryo (F); Heart stage (G); Torpedo stage (H) of *Jatropha curcas* L. Note : S = Suspensor, EP = Embryo proper

White yellow friable embryogenic calli were inoculated into cell suspension medium supplemented with 6,75 x 10^{-6} M 2,4-D and 4,4 x 10^{-7} M Kinetin. In this medium, single embryogenic cells were produced (Fig. 2 A). The single embryogenic cell is round and small, has dense cytoplasm,

large nucleus, little vacuole and thin cell wall. This single embryogenic cell divided within 5 days of culture as shown in Fig. 2 B. Fig. 2 C. shows embryo proper with suspensor. This embryo proper with suspensor often occurred in the cell suspension medium 7 days of culture. The existence of the suspensor gave the evidence that the embryo derived from a single cell. Mariani *et al*. (2002) reported that at an early stage, suspensors were observed on the elongated rice somatic embryo, because the somatic embryo was unicellular origin. Unicellular origin means that the somatic embryo was derived from a single cell. Suspensor in somatic embryo was also found in *Phaseolus vulgaris* (Puspitawati 1997), *Vigna radiata* (Puspitawati, 1997; Fitriani, 2002) *Allium sativum* (Nurwendah, 2002), *Lithospermum erythrorhizon* (Ramayanti, 2003; Mariani *et al*., 2004), and *Elaeis guineensis* (Wardjo, 2006). Thereafter, the embryo proper divided further and formed proembryogenic mass (PEM) after 10 days of culture (Fig. 2 D.). The PEM grew into proembryo (Fig. 2 E) and globular somatic embryo (Fig. 2 F) after 14 days of culture. Heart stage (Fig. 2 G) and torpedo stage somatic embryo (Fig. 2 H) were developed in embryo development medium after 14 days of culture.

According to our result, the somatic embryo of *Jatropha curcas* L. underwent direct somatic embryogenesis derived from single cell. This is coincide with Noerhadi (1988) that direct somatic embryogenesis was the formation of embryos from single cell. This is advantegous because it can reduce somaclonal variation. In addition, this advantage will be important in applying the somatic embryo in plant genetic transformation (Mariani *et al*., 2002).

After it was understood that the cells of *Jatropha curcas* enable to undergo somatic embryogenesis, embryogenic cell suspension (ECS) was established. The ECS was established within 2 weeks of culture in embryogenic cell suspension medium (ECSM) (Fig. 3). The composition of ECSM was JC medium supplemented with 1,35 x 10^{-5} M 2,4-D and 4,4 x 10^{-7} M Kinetin. The population of embryogenic cells in ECS was very uniform (Fig. 4).

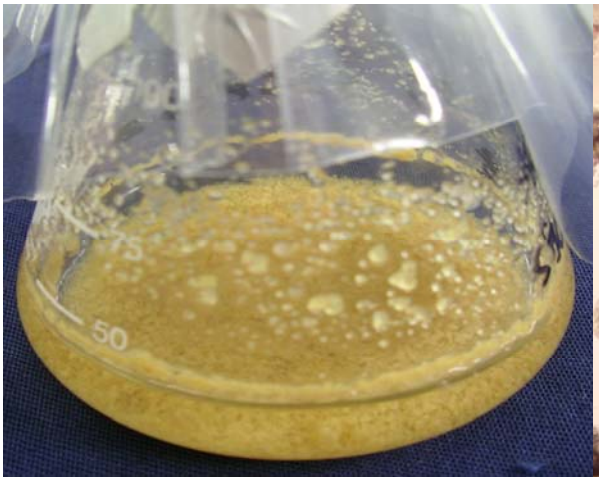

Fig. 3. Embryogenic cell suspension of *Jatropha curcas* L.

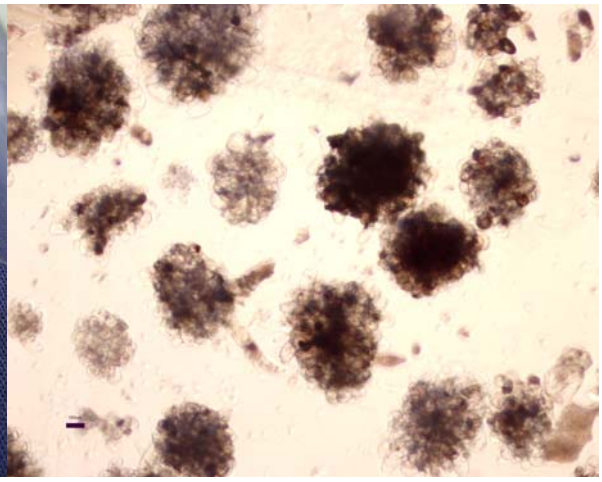

Fig. 4. Population of embryogenic cells of *Jatropha curcas* L. under inverted microscope

The ECS (Embryogenic cell suspension) is useful for protoplast isolation, micropropagation and as material for genetic transformation.

3. Plantlet development of *Jatropha curcas* L.

The plantlet developed from the embryogenic cells of *Jatropha curcas* L. after the ECS was cultured on embryo induction medium I, embryo induction medium II and embryo germination medium. Fig. 5. showed the plantlet of *Jatropha curcas* L.

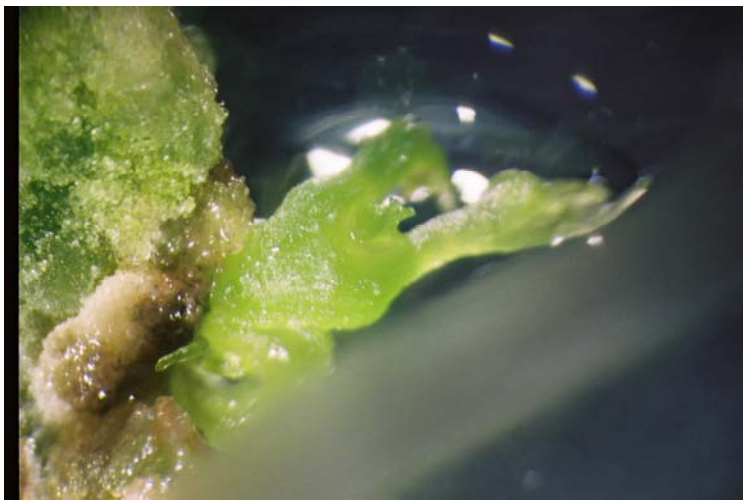

Fig. 5. Plantlet of *Jatropha curcas* on MS medium supplemented with 3ppm GA3 and 1ppm IAA

Germination of embryos were showed in fig. 6. The highest germination percentage was 63.3% on IG_4-gamborg medium. There was no germination on control medium. In germinated embryos, shoot and plantlet were still mixed. Therefore, observation on shoots and plantlet were performed and showed in fig. 7.

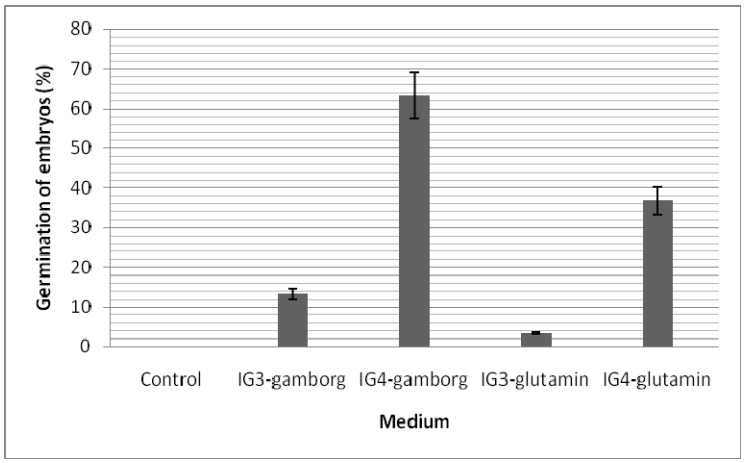

Fig. 6. Percentage of embryos germination on various embryo germination medium

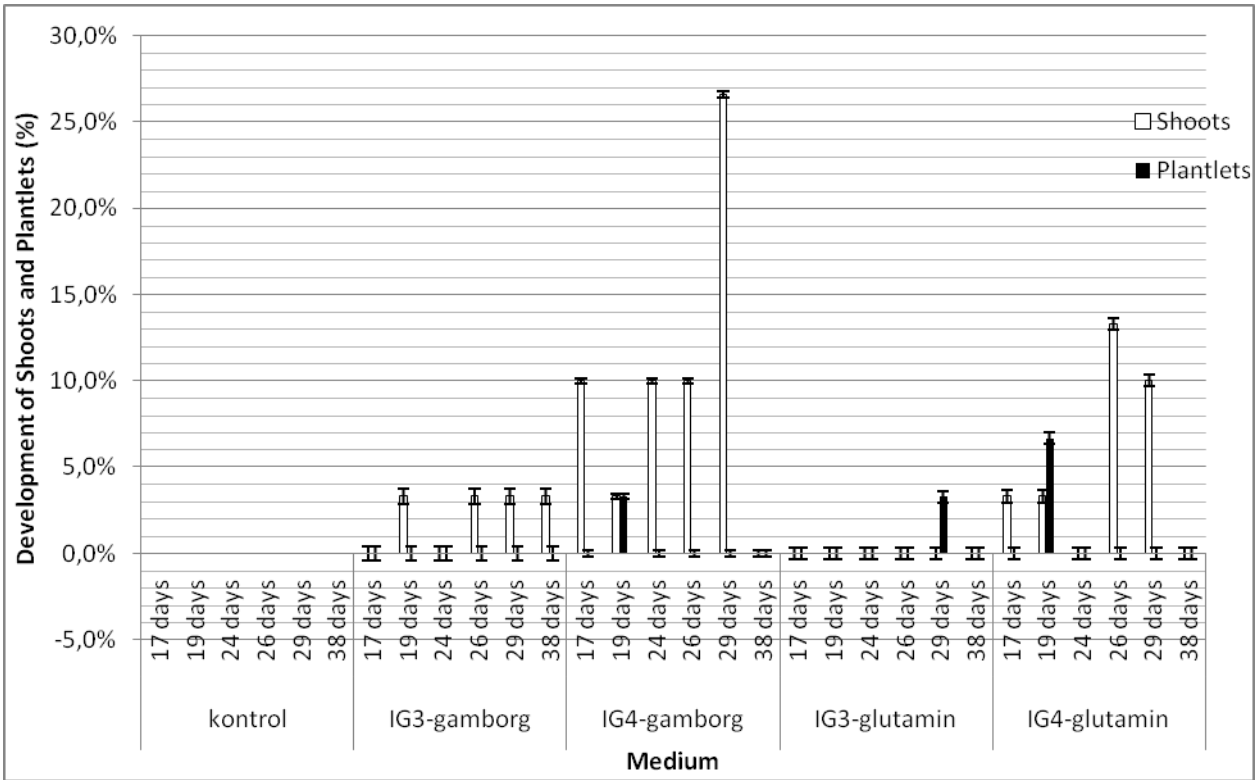

Fig. 7. Development of shoots and plantlets on various embryo germination medium

The highest shoot development was on IG_4-gamborg (60%) and the highest plantlet development was on IG_4-glutamin (6.7%). It was indicated that 1 ppm IAA and 3 ppm GA3 were suitable for the development of shoot and plantlet. Glutamin helped the development of plantlet on IG3-glutamin and Ig4-glutamin media.

Purnamaningsih (2002) reported that glutamine enhanced the development of embryo into torpedo and cotyledone stage. Others substances in glutamine medium such as citric acid as potassium chelating agent could increase somatic embryos amount and ascorbic acid as antioxidant could convert somatic embryos into plantlet (Herman, 2002).

4. Ultrastructural features

The percentage of plantlet development of *Jatropha curcas* L. was low (6.7%). Therefore, we observed the ultrastructural features of the cells, embryogenic cells and the somatic embryos by transmission electron microscopy. Fig 8. shows the ultrastructure features of cells suspension of *Jatropha curcas* L. It was clear that the vacuole was clean. Fig. 9 showed the ultrastructural features of embryogenic cells. It was prominent that the vacuoles contain many deposit material. Fig. 10. shows the ultrastructural features of somatic embryos. It indicated that the deposit material these deposits seem to form inside the tonoplast.

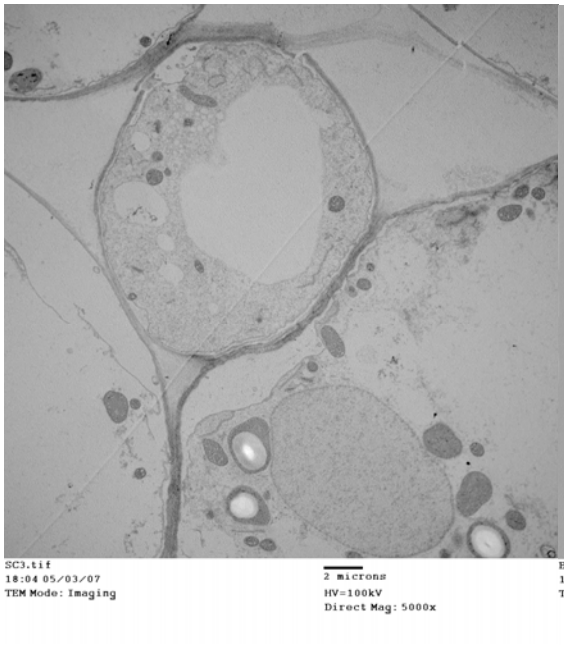

Fig. 8. Ultrastructural features of the cells of *Jatropha curcas* L.

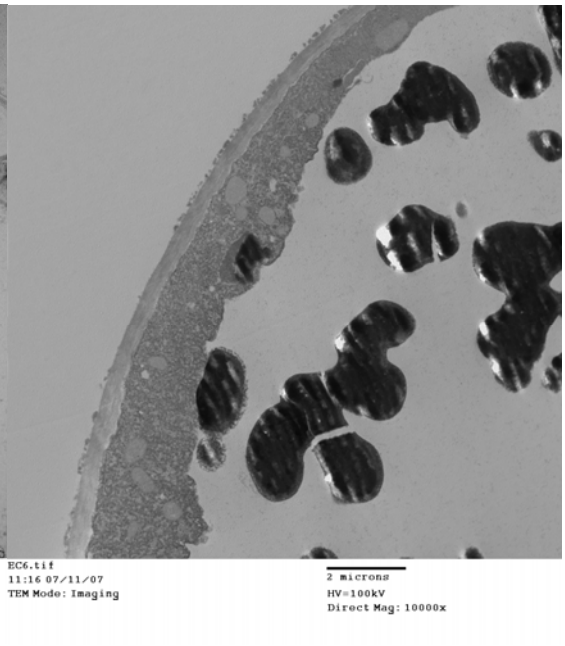

Fig. 9.Ultratsructural features of the embryogenic cells of *Jatropha curcas* L.

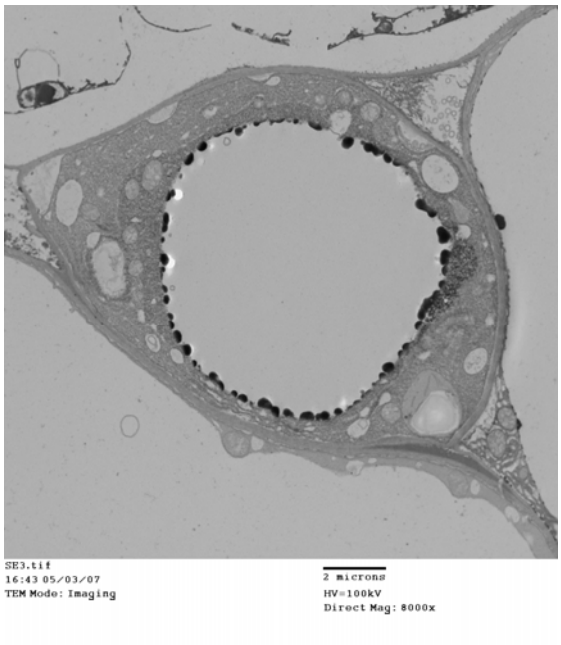

Fig. 10. Ultrastructural features of the somatic embryo of *Jatropha curcas* L.

Based on the observation of the ultrastructural features of the embryogenic cells and the somatic embryos, it was understood why the percentage of plantlet development was low. It was due to the deposit material in the vacuoles. The deposit material inhibited cellular metabolism of the cells. Therefore, only 6.7% of plantlet was developed from the embryogenic cells of *Jatropha curcas* L.

Conclusion

In conclusion, this is the first report of direct somatic embryogenesis of *Jatropha curcas* from single cells. Somatic embryos of *J. curcas* L. have been established in suitable liquid medium. The somatic embryos derived from single cell. This is advantageous because it can reduce somaclonal variation. In addition, this advantage will be important in applying the somatic embryo in plant genetic transformation. Plantlet of *J. curcas* developed from embryogenic cell suspension plated in solid medium. Character of the embryogenic cells and somatic embryo is forming deposit material in the vacuole.

References

1) Basubutra,R. and Sutiponpeibum, S. *Renewable energy. Review Journal.* 1982(4): 56-70.

2) Filho, J.C.B and Hattori, K. *R Bras Fisiol.Veg.* 1997. 9 (3): 185-188.

3) Fitriani, N. *Thesis Pascasarjana ITB*. 2002. Bandung.

4) Gupta, P.K., Timmis, R. and Carlson W. *Abstract of SIVB conference.* 2003.

5) Herman, Edwin B.. *Recent Advances in Plant tissue Culture VII. Regeneration and Micropropagation : Techniques, Media and Applications 1999-2002.* 2002. Agritech Consultant, Inc : USA

6) Mariani, T.S., Miyake, H. and Y. Takeoka. *Jurnal Matematika dan Sains.* 2002. 7(2): 53-56

7) Mariani, T.S., Ramayanti, O., Yazaki, K. and H. Miyake. *Annales Bogorienses*. 2004. 9(2):72-79.

8) Nigam, N., Saxena, O.P and Braganza, V.. *Trends in Plant Tissue Culture and Biotechnology.* 2005. Ed. Agrobios, India.

9) Noerhadi, E. *Seminar Bioteknologi Tanaman* 1988: 4

10) Nurwendah, I. *Skripsi Sarjana ITB* 2002. Bandung.

11) Purnamaningsih, R. *Buletin AgroBio.* 2002. 5(2):51-58

12) Puspitawati, R.P. *Tesis Sarjana ITB* 1997. Bandung.

13) Raina, A.K. and Gaikwad, B.R. *Oil JOTAI* 1987. Oct-Dec. 81-85

14) Ramayanti, O. Skripsi Sarjana ITB. 2003. Bandung.

15) Sardana, J., Batra, A., Ali, D.J. *Phytomorphology.* 2000. 50 (3/4) 239-42.

16) Touchet (de) B, Duval Y and Pannerier C. *Plant Cell Reports*. 1991. 10: 529-532

17) Wardjo, N.G. .*Skripsi Sarjana ITB*. 2005. Bandung.

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