Short communication

DEVELOPMENT OF SOMATIC EMBRYO IN *Lithospermum erythrorhizon* Siebb. et Zucc AND THE STUDY ON THE EFFECT OF METHYL JASMONATE ON ITS MATURATION

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ABSTRACT

A research on the somatic embryogenesis in *Lithospermum erythrorhizon* has been conducted. Embryogenic callus was inoculated in EIM9 liquid medium (modification of LS medium), i.e. L2PVP (initiation medium) and the development of somatic embryo was observed. Ten µM and 100 µM methyl jasmonate was added into L3PVP medium (differentiation medium) for somatic embryo maturation. The purposes of this research were to observe the development of somatic embryo and to observe the effect of methyl jasmonate on maturation of the somatic embryo in *L. erythrorhizon*. The results showed that somatic embryogenesis in *L. erythrorhizon* derived from single cells differentiated further forming proembryo, globular, heart, torpedo and cotyledon stage. Treatment with 10 µM and 100 µM methyl jasmonate induced maturation of somatic embryos (cotyledon stage) after transferring them to embryo development medium (L4PVP).

Keywords: Somatic embryogenesis, *Lithospermum erythrorhizon*, methyl jasmonate.

ABSTRAK

Telah dilakukan penelitian mengenai embriogenesis somatik pada *Lithospermum erythrorhizon*. Kalus embriogenik diinokulasikan pada medium cair EIM9 (modifikasi medium LS), yaitu L2PVP (medium inisiasi) dan perkembangan embrionya diamati. Metil jasmonat sebanyak 10 µM dan 100 µM ditambahkan pada medium L3PVP (medium diferensiasi) untuk percobaan maturasi embrio somatik. Tujuan penelitian ini adalah untuk mengamati perkembangan embrio somatik dan untuk mengamati pengaruh metil jasmonat pada maturasi embrio somatik *L. erythrorhizon*. Hasil percobaan menunjukkan bahwa embriogenesis somatik pada *L. erythrorhizon* berasal dari sel tunggal yang berdiferensiasi lebih lanjut membentuk proembrio, embrio tahap globular, hati, torpedo dan kotiledon. Perlakuan dengan metil jasmonat 10 µM dan 100 µM dapat menginduksi maturasi embrio somatik (tahap kotiledon) setelah memindahkannya ke medium perkembangan embrio (L4PVP).

Kata kunci: *Embriogenesis somatik, Lithospermum erythrorhizon, metil jasmonat.*

INTRODUCTION

Lithospermum erythrorhizon Sieb. et Zucc. is a traditional medicinal plant belong to Boraginaceae that has been used worldwide. *L. erythrorhizon* produces antibacterial, antifungi, wound-healing, anti-inflammatory, analgesic, anti-tumor compounds and can be used as stimulant that activates immune response (Bown, 1995; Yazaki *et al.*, 1999). Due to its importance and its indiscriminate collection from the wild, *L. erythrorhizon* is a rare and endangered plant in Japan (Fowler, 1983; Yazaki *et al.*, 1997a).

The distribution area of *L. erythrorhizon* is restricted to East China, Japan and Korea. Today, this plant is rarely found and therefore needs to be conserved (Bown, 1995). Natural condition in Indonesia makes it possible to grow *L. erythrorhizon* so that a preliminary study for the development of this plant in Indonesia could be conducted.

In vitro culture method of *L. erythrorhizon* is conducted for the preliminary study. Korean researcher carried out plant regeneration from callus culture of *L. erythrorhizon* through organogenesis and somatic embryogenesis. However, morphologically regeneration mostly has been conducted through organogenesis (Yu *et al.*, 1997). Therefore, for the first time, somatic embryogenesis of *L. erythrorhizon* in liquid culture is conducted.

Methyl jasmonate (MeJa) inhibits germination and growth, promotes senescence, induces tuber formation and functions as wound-inducible signaling pathway in various plant systems (Tokuji *et al.*, 1995). According to research on carrot somatic embryo, regeneration of plantlets was markedly inhibited by the presence of 1 mM MeJa and was essentially absent in the presence of 20 mM MeJa (Tokuji *et al.*, 1995). In particular, MeJa inhibits the germination of lettuce seed, a property that seems to be relevant to our study of somatic embryogenesis. Their result showed that embryogenic cell clusters cultured in liquid medium containing MeJa developed up to torpedo stage. However, further development to form plantlet was inhibited. During the inhibition process, the carrot's somatic embryo became mature and the torpedo embryo developed into plantlet in the liquid medium without MeJa (Tokuji *et al.*, 1995).

In this report, we describe the development of somatic embryo using liquid culture and the effect of methyl jasmonate on the maturation of somatic embryo in *L. erythrorhizon*.

MATERIALS AND METHODS

Materials

Internodes of *in vitro* shoot cultures were used as the explants for embryogenic calli induction. The internodes were cut into several segments, and then put on calli induction medium (CIM). The CIM consisting of MS Basal medium (Murashige & Skoog, 1962) supplemented with 10 mM NAA, 10 mM Kinetin, 3% sucrose and solidified with 0.8% agar. The cultures were kept in the dark at 25°C. The yellowish friable calli obtained were then used as a source of inoculum for embryogenic cell culture.

Methods

Embryogenic callus culture *in vitro* was maintained in embryogenic callus initiation medium (EIM9) (Table 1). EIM9 medium is M9 Medium (Yazaki *et al.*, 1997b), a modified Linsmaier & Skoog (LS) medium (Linsmaer & Skoog, 1965). Four colonies of calli were placed in the media in a plastic Petri dish sealed with parafilm. The cultures were placed in dark culture room, at 25°C. Four weeks old calli were subcultured onto fresh EIM9 medium.

To initiate embryogenic cell culture and somatic embryogenesis, 2-3 g of 8 week-old embryogenic calli were inoculated into

20 ml L2PVP medium (Table 1). Cell suspension cultures were agitated on a reciprocal shaker (80 rpm), maintained for a week in the dark at 25°C. Embryogenic cells were subsequently proliferated in L2PVP liquid medium. Single embryogenic cells, dividing cells and proembryos were subcultured into fresh L2PVP medium and observed for up to 10 d. Somatic embryo cultures were maintained in L2PVP medium for 3 weeks. Subsequently, synchronization was conducted using 1 mm nylon mesh.

For maturation, 2 week-old synchronized somatic embryos (globular, early heart and heart embryos) were transfered into L3PVP media (Table 1) containing 10 µM and 100 µM MeJa and maintained for 10 d. Thereafter, somatic embryos were subcultured into 20 ml L4PVP medium (Table 1) and maintained for 20-24 d. As a control, somatic embryos in L2PVP medium were subcultured into L3PVP medium without MeJa for 10 d. Subsequently, the cultures were subcultured into L4PVP medium. All flasks were agitated on a reciprocal shaker (80 rpm), in the dark at 25°C.

Observation of somatic embryo development in L2PVP, L3PVP and L4PVP medium was conducted every 2-3 d for 6 weeks by using inverted microscopy.

RESULTS AND DISCUSSION

Development of *L. erythrorhizon* somatic embryo

Somatic embryogenesis in *L. erythrorhizon* derived from single cells underwent different stages of development; proembryo, globular, heart, torpedo and cotyledon stages. Callus of *L. erythrorhizon* was soft, yellowish with delicate nodules in the surface of callus (Figure 1). Cells in the delicate nodules were supposed to actively proliferate and to form meristematic cells cluster; which have the potency to become embryogenic cells in an appropriate medium (Filho & Hattori, 1997).

Figure 1. Embryogenic callus of *L. erythrorhizon*

Table 1. Medium for somatic embryogenesis of *L. erythrorhizon*

Medium	Proline (g/L)	Carbon source (%)		Hormones (µM)		Gelling agent Phytagel (%)	PVP (%)
		Sucrose	Sorbitol	Kinetin	NAA		
EIM9	1.5	3	0	10	10	0.25	0
L2PVP	1.5	3	0	0.5	5	0	0.3
L3PVP	1.5	3	0	5	5	0	0.3
L4PVP	1.5	2	4	5	1	0	0.3

Somatic embryo developed very fast after 3 d in L2PVP medium and formed proembryo from embryogenic callus. Decreasing NAA concentration to 5 μM and kinetin to 0,5 μM in L2PVP medium was to initiate somatic embryo formation from embryogenic calli. Somatic embryogenesis occurred on high auxin and low cytokinin concentrations (Duval *et al.*, 1995). Embryogenic cell culture was viscous, because the extracellular matrix (polysaccharides) was excreted into the medium (Jayasankar *et al.*, 1999).

Proembryo was formed on the 3rd day of culturing in L2PVP medium. This proembryo was derived from a single cell that was actively dividing longitudinally to become 2 cells, 4 cells, and so on until proembryo was formed (Fig. 2a-2f). The formation of proembryo from embryogenic callus indicated that somatic embryo induction of *L. erythrorhizon* occurred indirectly.

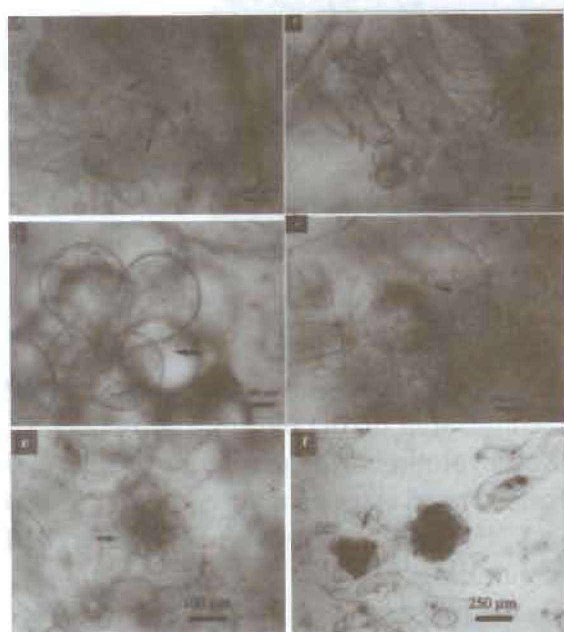


Figure 2. Early induction of somatic embryo of *L. erythrorhizon*

a. Single cells.
b. Asymmetric division (2 cells).
c. Symmetric division (4 cells).
d. Further division with suspensor.
e. Further division without suspensor.
f. Proembrio.

A single cell (Fig. 2a) appeared to be small and round with dense cytoplasm surrounded by a thin cell wall. Observation of the initiation of somatic embryo indicated that there were 2 kinds of divisions, i.e. asymmetry that resulted in 2 cells with different sizes (Fig. 2b) and symmetry that resulted in 4 cells with the same sizes (Fig. 2c). In asymetric division, one cell received more cytoplasm, whereas another cell vacuolated. The cell containing cytoplasm divided longitudinally and transversally to become proembryo (p) (Fig. 2f). The vacuolated cell divided to form a small filament called a suspensor (s) (Fig. 2d). After 4-cell stage, daughter cells divided further (Fig. 2e) to become 8 cells, 16 cells and so on until proembryo was formed (Fig. 2f). Cells that divide further have small sizes and dense cytoplasms.

The suspensor of the somatic embryo was formed due to the existence of a suspensor gene. However, its mechanism have not yet known (Puspitawati, 1997). Suspensor of the somatic embryo was also found in *Phaseolus vulgaris* (Fitriani, 2002), *Vigna radiata* (Puspitawati, 1997; Fitriani, 2002) and *Allium sativum* (Nurwendah, 2002). In somatic embryo of *Phaseolus vulgaris*, the suspensor has a role in nutrition absorption from the growth medium for embryo development (Fitriani, 2002). The role of suspensor in the somatic embryo of *L. erythrorhizon* might be the same as that of *Phaseolus vulgaris*.

On the next stage of development, the proembryo formed a globular somatic embryo (Fig. 3). Observation of the globular somatic embryo in the 10th days of culture in L2PVP medium showed that it had a suspensor (Fig. 3b). Besides globular embryos with suspensors, generally globular embryos without suspensors were also found (Fig. 3a). In the globular stage, the whole cells

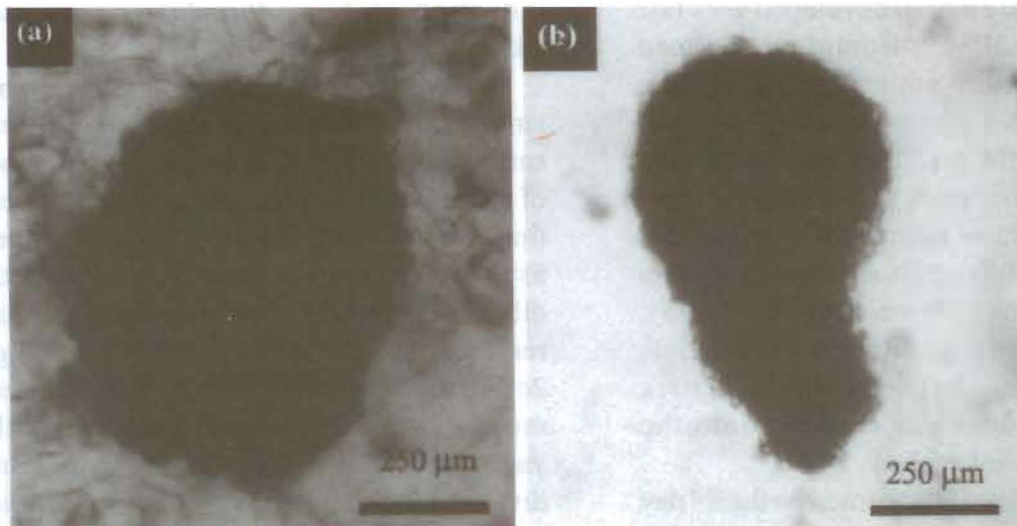


Figure 3. Globular somatic embryo of *L. erythrorhizon* (a) without suspensor; (b) with suspensor

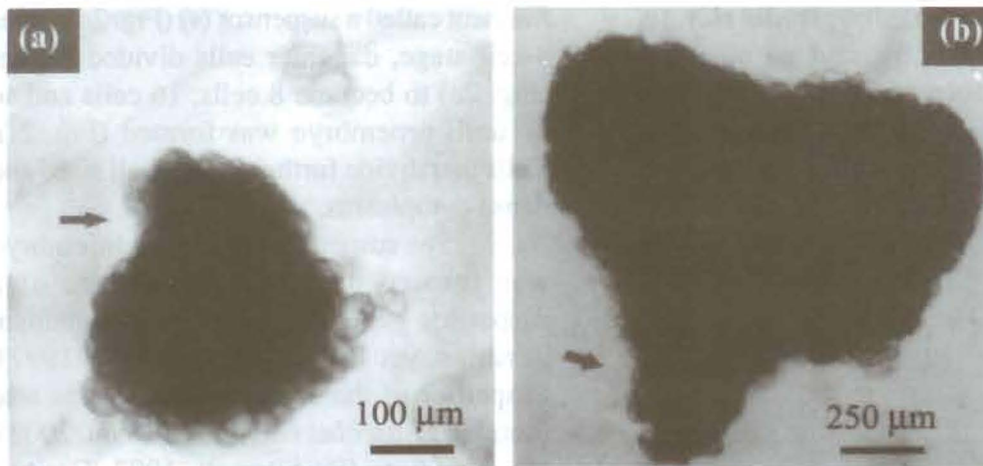


Figure 4. Early heart somatic embryo (a) and heart somatic embryo (b) of *L. erythrorhizon*

are morphologically similar and protoderms can be differentiated (Fosket, 1994).

Following the transfer into L3PVP medium, the globular somatic embryo underwent a development into a heart stage embryo (Fig. 4). In the L3PVP medium, the concentration of NAA and kinetin was the same (5 µM) (Table 1). Equal concentration of the two plant growth regulator was needed for somatic embryo differentiation in palm oil (Duval *et al.*, 1995). Observation showed that there was a plateau on embryo axis of globular somatic embryo that was found before the embryo developed into heart stage embryo. This phase was an early heart stage embryo (Fig. 4a). This early heart stage somatic embryos were found in the L3PVP medium after 6 d of culturing. Subsequently, the development of cotyledon primordial formed heart stage somatic embryo (Fig. 4b) in L3PVP medium was obtained after 10 d of culturing.

Methyl Jasmonate treatment for embryo maturation

Methyl Jasmonate (MeJa) treatment influenced the developmental stage of *L. erythrorhizon* somatic embryo. MeJa induced the development of somatic embryo until early torpedo stage when the somatic embryo was transferred into L3PVP medium

containing 10 μM MeJa (Fig. 5a). In L3PVP medium containing 100 μM MeJa, torpedo somatic embryo was obtained (Fig. 5b). Similar result was obtained in carrot somatic embryogenesis. One μM and 100 μM of MeJa increased the developmental stage of carrot somatic embryo up to torpedo stage (Tokuji *et al.*, 1995). However, MeJa inhibited further differentiation of torpedo somatic embryo to become cotyledonary somatic embryo.

To detect the maturity of the somatic embryo after a treatment with 10 μM MeJa in L3PVP medium, the somatic embryos (globular, early heart, and heart stage) were transferred to embryo development medium (L4PVP). The result showed that very few torpedo and cotyledon were obtained after 5 weeks of culturing (Figure 6). Likewise, after

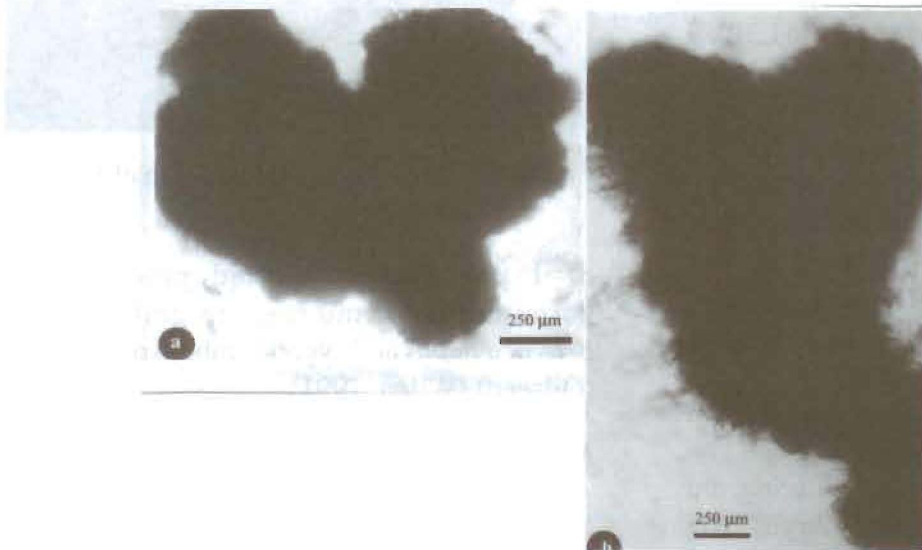
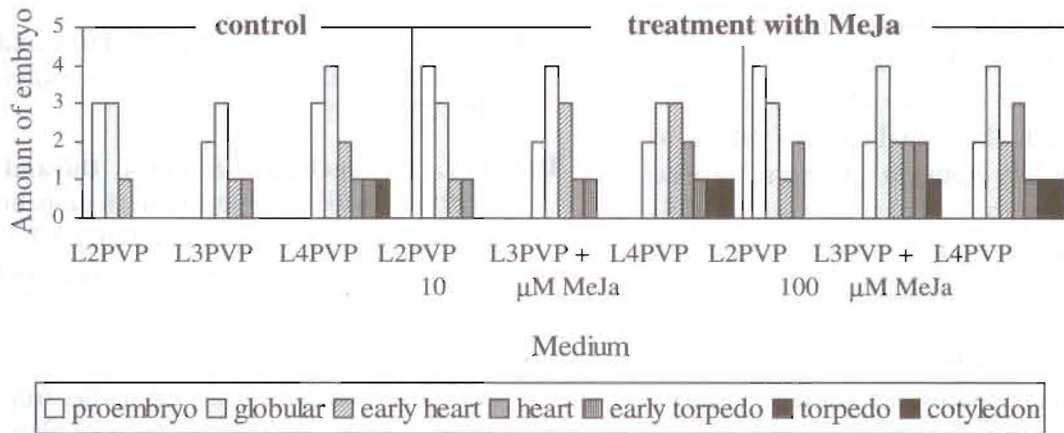


Figure 5. Early torpedo somatic embryo (a) and torpedo somatic embryo (b) of *L. erythrorhizon*



Note on number of embryo:

1 = very few (< 5 embryos)
2 = few (6 – 10 embryos)
3 = moderate (11 – 20 embryos)
4 = abundant (21 – 40 embryos)

Figure 6. Production of somatic embryo with MeJa treatment for embryo matur tion. The inoculum were grown on EIM9 (2x subcultures), 10 d on L2PVP and 14 d on I 3PVP

treatment with 100 μM MeJa in L3PVP medium, the somatic embryos (globular, early heart and heart stage) were transferred to L4PVP medium. The result showed that very few cotyledons (2 cotyledons) was obtained on the 5th days of culture (Figure 6). Similar result was reported on carrot somatic embryos treated with MeJa. The carrot torpedo somatic embryos developed into cotyledon somatic embryos after transfering into medium without MeJa (Tokuji *et al.*, 1995).

Treatment of 100 μM MeJa in L3PVP medium resulted in more heart stage embryos (15 embryos) compared to those of heart stage embryos (8 embryos) obtained in L3PVP medium containing 10 μM MeJa (Figure 6). On the 6th week of culturing, the amount of cotyledon embryos in L4PVP medium that previously treated with 100 μM MeJa in L3PVP medium increased to 4 mature cotyledon embryos.

Different result was obtained from L4PVP medium without MeJa treatment. The somatic embryos; 6 weeks after culturing, did not produce mature cotyledon embryos. The development of somatic embryos only achieved a few torpedo stage embryos (Figure 6).

Harvested cotyledon somatic embryo has a fully expanded cotyledon showing the maturity of the embryo (Figure 7). It is suggested that the capability of MeJa to induce secondary embryos (heart and torpedo somatic embryos) (Tokuji *et al.*, 1995) influenced further differentiation to achieve a mature cotyledon somatic embryo at the MeJa concentration of 100 μM.

In the present study, it is shown that precocious germination of somatic embryo of *L. erythrorhizon* was inhibited by the addition of MeJa (10 μM and 100 μM). Treatment of MeJa induced further maturation of somatic embryos (cotyledon stage) after transferring them to embryo development medium.

Similar results were obtained from other studies; which showed that the addition of 1 μM MeJa inhibited precocious germination in microspore embryo of *Brassica napus* and zygotic embryo of Linum (Oilseed) (Wilen, 1991).

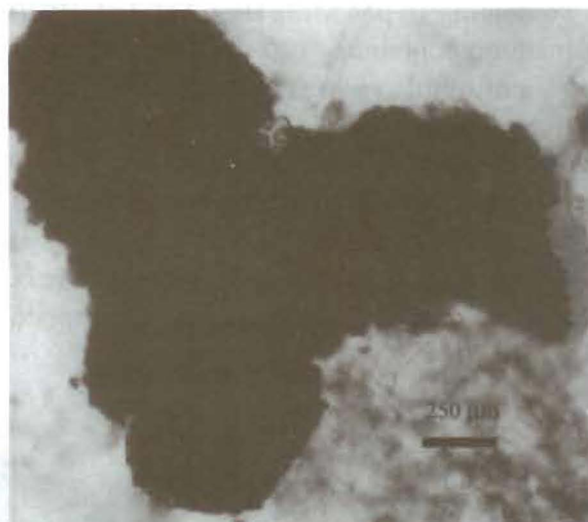


Figure 7. Cotyledon somatic embryo of *L. erythrorhizon*

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REFERENCES

Bown, D. 1995. Encyclopedia of Herbs and Their Uses. Dorling Kindersley, London. Pp. 238 – 239.

Duval, Y., F. Engelmann & G.T.T. Durand. 1995. Somatic embryogenesis in oil palm (*Elaeis guineensis*, Jacq.) In: Bajaj, Y.P.S. (Ed). *Biotechnology in Agriculture and Forestry*. Berlin. Springer – Verlag. Berlin.

Filho, J.C.B & K. Hattori. 1997. Embryogenic callus formation histological studies from *Stevia reboudiana* (Bert.) Bertoni floret explants. *R. Bras. Fisiol. Veg.* 9 (3): 185-188.

Fitriani, N. 2002. Perkembangan Embrio Somatik dari Kotiledon Kacang Hijau (*Vigna Radiata* (L) Wilczek). Tesis pascasarjana ITB. Bandung.

Fosket, D.E. 1994. *Plant Growth and Development: A Molecular Approach.* Academic Press, Inc., San Diego.

Fowler, M.W. 1983. Commercial application and economic aspects of mass plant cell culture. In: *Plant Biotechnology*. Mantell, S. H. & Smith, H. (Eds). Cambridge University Press. London.

Jayasankar, S., D.J. Gray & R.E. Litz. 1999. High efficiency somatic embryogenesis and plant regeneration from suspension cultures of grapevine. *Plant Cell Rep.* 18: 533-537.

Linsmaier, E. & F. Skoog. 1965. Organic growth factors requirements of tobacco tissue cultures. *Physiol. Plant.* 18: 100-127.

Murashige, T. & F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant.* 15: 473-497.

Nurwendah, I. 2002. Pengaruh 2,4-D terhadap embriogenesis somatik tak langsung dan perubahan struktur permukaan embrio somatik pada bawang putih (*Allium sativum* L.) kultivar Lumbu Hijau. Skripsi Sarjana ITB. Bandung.

Puspitawati, R. P. 1997. Embriogenesis pada Kacang Hijau (*Vigna radiata*) varietas walet. Tesis Sarjana ITB. Bandung.

Tokuji, Y., Y. Mizue & H. Masuda. 1995. Effect of methyl jasmonate and concavalin A on embryogenesis and the induction of secondary embryos of carrot. *Biochem.* 59 (9): 1675–1678.

Wilen, R.W. 1991. Effects of jasmonic acid on embryo specific processes in Brassica and Linum oilseeds. *Plant Physiol.* 98(2): 399-405.

Yazaki, K., H. Fukui, Y. Nishikawa & M. Tabata. 1997a. Measurement of phenolic compounds and their effect on shikonin production in *Lithospermum* cultured cells. *Biosci. Biotech. Biochem.* 61 (10): 1674–1678.

Yazaki, K., K. Takeda & M. Tabata. 1997b. Effects of methyl jasmonate on shikonin and dihydroechinofuran production in *Lithospermum* cell cultures. *Plant Cell Physiol.* 38 (7): 776–782.

Yazaki, K., H. Matsuoka, T. Ujihara & F. Sato. 1999. Shikonin biosynthesis in *Lithospermum erythrorhizon*: light – induced negative regulation of secondary metabolism. *Plant Biotechnol.* 16 (5): 335–342.

Yu, H. J., S.Y. Oh, M.H. Oh, D.M. Choi, Y.M. Kwon & S.G. Kim. 1997. Plant regeneration from callus cultures of *Lithospermum erythrorhizon*. *Plant Cell Rep.* 16 (5): 261–266.