

Effect of 2,4-D on Indirect Somatic Embryogenesis and Surface Structural Changes in Garlic (*Allium sativum* L.) cv. Lumbu Hijau

Totik Sri Mariani¹⁾, Hiroshi Miyake²⁾, Rizkita Rachmi Esyanti¹⁾, and Ida Nurwendah³⁾

¹⁾Department of Biology, Faculty of Mathematics and Natural Sciences,
Institut Teknologi Bandung, Jalan Ganesa 10, Bandung 40132, Indonesia

²⁾Graduate School of Bioagricultural Sciences, Nagoya University,
Nagoya 464-8601, Japan

³⁾Sekolah Tinggi Farmasi, Bandung, Indonesia
email: totik@bi.itb.ac.id

Received August 2003, accepted for publication October 2003

Abstrak

Telah dilakukan penelitian tentang pengaruh 2,4-D terhadap embriogenesis tak langsung dan perubahan struktur permukaan embrio somatik pada bawang putih (*Allium sativum* L.) kultivar Lumbu Hijau. Tujuan penelitian ini untuk mengetahui pengaruh 2,4-D pada proses induksi embriogenesis somatik serta mempelajari tahap-tahap perkembangan embrio somatik dan pengamatan perubahan struktur permukaan embrio somatik bawang putih. Eksplan ujung akar ditanam pada medium induksi kalus embriogenik (ECIM). Embrio somatik selanjutnya dipindahkan pada medium maturasi embrio (EMM), kemudian didesikasi, dan selanjutnya dipindahkan pada medium embriogenesis somatik repetitif (MESR). Pengamatan dengan menggunakan mikroskop diseksi menunjukkan bahwa pembentukan kalus embriogenik dan embrio somatik terjadi pada MIKE yang mengandung 0.1 μM 2,4-D. Pada MME dengan konsentrasi 0,01 μM 2,4-D, embrio somatik globular berkembang menjadi embrio somatik matang. Pada MESR tanpa zat pengatur tumbuh, embrio somatik mengalami embriogenesis somatik repetitif yang mengandung embrio globular dan embrio matang. Pengamatan dengan "scanning electron microscopy" (SEM) memperlihatkan bahwa tahap-tahap embriogenesis somatik pada bawang putih meliputi tahap proembrio, tahap transisi dari proembrio ke tahap globular, tahap globular, tahap embrio matang dan tahap kotiledon tunggal. Pengaruh 2,4-D terhadap perubahan struktur permukaan dapat dilihat dari setiap tahap perkembangan embrio somatik. Pada 0,1 μM 2,4-D terbentuk proembrio, embrio transisi dan embrio globular. Permukaan proembrio halus, sedangkan permukaan embrio somatik tahap transisi diliputi oleh materi fibrillar berupa mikrofibril selulosa. Pada tahap globular, seluruh permukaan embrio telah ditutupi oleh dinding sel yang baru. Selanjutnya, pada 0,01 μM 2,4 D terbentuk embrio matang dan kotiledon tunggal, dimana permukaan dinding selnya telah stabil. Dari penelitian ini dapat disimpulkan bahwa konsentrasi 2,4-D yang optimum untuk induksi kalus embriogenik dan inisiasi embrio somatik adalah 0,1 μM . Pengurangan konsentrasi 2,4-D menjadi 0,01 μM menyebabkan embrio berkembang lebih lanjut menjadi tahap matang dan kotiledon tunggal. Pada medium MESR tanpa zat pengatur tumbuh terjadi embriogenesis somatik repetitif. Pada penelitian ini terlihat bahwa struktur permukaan embrio sangat khas pada setiap tahap perkembangan embrio somatik.

Kata kunci : Bawang putih, embriogenesis somatik, 'scanning electron microscopy'

Abstract

A study concerning the effect of 2,4-D on indirect somatic embryogenesis and surface structural changes in garlic (*Allium sativum* L.) cv. Lumbu Hijau was conducted. The purposes of this study were to evaluate the effect of 2,4-D on the induction of somatic embryogenesis and to observe the developmental stage of somatic embryo as well as the surface structural changes of somatic embryo in garlic. Root tips explants were cultured on embryogenic callus induction medium (ECIM). Somatic embryos were then transferred to the embryo maturation medium (EMM), desiccated and subsequently transferred to the repetitive somatic embryogenesis medium (RSEM). Observation by dissecting microscope showed that embryogenic callus and somatic embryo was formed at ECIM containing 0.1 μM 2,4-D. At EMM containing 0.01 μM 2,4-D somatic embryo developed into mature somatic embryo. Somatic embryo underwent repetitive somatic embryogenesis that consisted of globular and mature somatic embryo at RSEM, without plant growth regulator. Observation by scanning electron microscopy showed that the developmental stages of garlic somatic embryo consisted of proembryo, transition phase from proembryo to globular, globular, mature embryo and single cotyledon stages. Effect of 2,4-D on the cell surface structure could be seen on each somatic embryo developmental stage. In ECIM containing 0,1 μM 2,4-D, proembryo, transition embryo and globular embryo were formed. The surface of proembryo was smooth whereas fibrillar material as cellulose microfibril, was observed during the transition from proembryo to globular stage. The surface of globular stage was entirely covered

by a new cell wall. Subsequently, in 0.01 μM 2,4-D, mature embryo and single cotyledon were formed, which their cell wall surface was stable. From this study, it could be concluded that the optimum concentration of 2,4-D for induction of embryogenic callus and initiation of somatic embryo was 0.1 μM . Decreasing of 2,4-D concentration to 0.01 μM resulted in the development of globular embryo to mature and single cotyledon stage. Repetitive somatic embryogenesis occurred in RSEM medium without plant growth regulator. Based on this result it can be concluded that each somatic embryo developmental stage had a typical characteristic of surface structure.

Keywords: Garlic, somatic embryogenesis, 'scanning electron microscopy'

1. Introduction

Garlic (*Allium sativum* L.) is a worldwide crop used for both culinary and medicinal purposes¹⁾. In Indonesia, garlic has been used as a traditional medicine, especially to cure several diseases such as TBC, influenza, diabetes, high blood pressure and rheumatismo²⁾. In agriculture, garlic can be used as bactericide and fungicide³⁾.

Indonesia still imports a large quantity of garlic due to the lack of good qualities of garlic seeds. 'Lumbu Hijau' is one of the top cultivar in Indonesia. Garlic cv. Lumbu hijau is a highland cultivar, which has growth period faster than that of lowland cultivar. Plant propagation through somatic embryogenesis is needed to produce good qualities of garlic seeds.

Somatic embryogenesis is advantageous for mass propagation, genetic improvement programs and production on synthetic seeds⁴⁾. Somatic embryogenesis on the *A. sativum* L., cv. 'Lumbu Hijau' has never been reported. Bangladesh researcher reported the efficient plant regeneration in garlic, but in another cultivar (*A. sativum* L., 'White roppen') through somatic embryogenesis⁵⁾. It has been known that the genotype also influence the regeneration potential of cultured plant^{6,7)}. Therefore, we studied somatic embryogenesis of *A. sativum* L., cv. 'Lumbu Hijau'.

Somatic embryogenesis can be used as a model to study the physiology and morphology of the somatic embryo as well. Therefore, in the present study, we evaluated the effect of 2,4-D on the induction of somatic embryogenesis as well as observed the morphological changes of the surface structure during somatic embryogenesis. The cell surface is the entire assembly of cell wall, plasma membrane and cortical cytoskeleton. The dynamic and integrated assembly as a whole is now considered to play a vital and central role in defining cell shape, cell polarity and morphogenesis in plants⁸⁾.

2. Materials and Methods

2.1 Material

Root tips of garlic (*Allium sativum* L. cv. 'Lumbu Hijau') were used as an explant for somatic embryogenesis.

2.2 Methods

2.2.1 Root tips formation

Cloves of garlic (*A. sativum* L. cv. 'Lumbu Hijau') was sterilized by immersion, first in 70% ethanol for 30 s and then in 1% sodium hypochlorite solution containing Tween 20 (2 drops/ 100 ml solution). Each clove then was sprouted aseptically in a culture flask containing 25 ml water-agar (2.5 g L⁻¹ phytagel in distilled water). The flask was covered with polypropylene closures, sealed with Parafilm and incubated in continuous light (15 $\mu\text{ moles m}^{-2} \text{ s}^{-1}$) at 28°C for 10 to 15 days. Root tips were excised from these sprouted cloves.

2.2.2 Embryogenic calli induction and somatic embryo formation

Root tip (2-3 mm) was excised aseptically. Five root tips were cultured on sterile plastic Petri dish containing 25 ml embryogenic callus induction medium (ECIM). Table 1 shows the composition of media. Firstly, the experiment was carried out using the medium listed in the table 1 for optimization. The best media for inducing embryogenic calli and somatic embryo formation was then used. The optimized medium consisted of three replications and each replication comprised of 50 explants. Routine observations were done using a stereo microscope.

2.2.3 Somatic embryogenesis maturation and repetitive

Somatic embryo containing-calli were transferred on embryo maturation medium (EMM) (Table 1). The best EMM was then applied for maturation of somatic embryo. The mature somatic embryos were then desiccated to prevent precocious germination. For desiccation treatment, the mature somatic embryos were desiccated on two layers of sterilized filter papers in petri dish for 24 h. Subsequently, the desiccated somatic embryo was transferred onto repetitive somatic embryogenesis medium (RSEM)

Table 1. The composition of media for producing garlic somatic embryos.

Medium	Basal Medium	Plant hormone (2,4-D) μM	Carbon source		Gelling agent (Phytigel)
			Sucrose	Sorbitol	
ECIM	MS ^{*)}	0.1	3%	-	0.25%
		0.5			
		1.0			
		5.0			
EMM	MS	0.05	2%	4%	0.25%
		0.1			
		0.5			
		1			
RSEM	MS	-	3%	-	0.25%

ECIM and EMM were supplemented with 1.5 g L^{-1} proline

^{*)} Murashige and Skoog (1962)⁹⁾

2.2.4 Scanning electron microscopy (SEM)

The morphogenic development of somatic embryos was examined by SEM. For the SEM study, calli that contain somatic embryos at different developmental stages were fixed with 5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 at 4°C for 24 h. Then, samples were rinsed in 0.1 M cacodylate buffer 2 times and once in aquadest. Thereafter, the samples were gradually dehydrated in an alcohol series; immersed in isoamyl acetate for 5 min and dried at critical point using CO₂ as the transient fluid. After critical point drying by DCP-1 (Denton Vacuum), the samples were coated with gold by an ion sputtering apparatus Jeol JEE and observed by SEM Philips XL 20.

3. Results and Discussion

3.1 Root induction

Growth of root was initiated with the formation of root projection at the base of garlic disc 1 to 2 days of culture. Thereafter, root projection elongated and was ready to be used as an explant for somatic embryogenesis at 10th days of culture (Fig.1).



Fig.1. Root (arrow) as the explant source of 10 days of culture

3.2 Embryogenic callus induction and somatic embryo formation

Embryogenic callus induction was conducted on embryogenic callus induction medium (ECIM) containing 0.1; 0.5; 1.0 or 5.0 μM 2,4-D. The observation using dissecting microscopy showed that the explant gave different response after 6 weeks of culture (Table 2).

Table 2. Response of 2,4-D on root tips explant of garlic

Concentration of 2,4-D μM	Response
5.0	Not growing
1.0	Callus without embryo
0.5	Callus without embryo
0.1	Callus with embryo

Explant could not grow at all on ECIM containing 5.0 μM 2,4-D. On ECIM containing 1.0 μM 2,4-D and 0.5 μM 2,4-D yellowish callus formed without somatic embryo, whereas on ECIM containing 0.1 μM 2,4-D white yellowish callus with the globular embryo on its surface was formed (Fig. 2). The occurrence of globular structure on the surface of callus was a sign of somatic embryogenesis¹⁰⁾. In the present study, the somatic embryogenesis was indirect because the callus was formed first and the somatic embryo was formed afterward. As stated by Fransz (1998) that indirect somatic embryogenesis is characterized by the occurrence of callus first¹¹⁾.

The result showed that 0.1 μM 2,4-D was the optimum concentration for embryogenic callus and somatic embryo induction on the root tip explant of garlic cv. Lumbu Hijau. This result showed that the optimum concentration of 2,4-D used was lower than other research on somatic embryo in *Allium*

spp. The optimum concentration of 2,4-D for embryogenic callus and somatic embryo induction on the garlic cv. White Roppen was $0.5 \mu\text{M}$ ⁵. It is proved that the requirement of 2,4-D to induce the somatic embryogenesis was varying due to the difference in genetic factor^{6,12,7}.

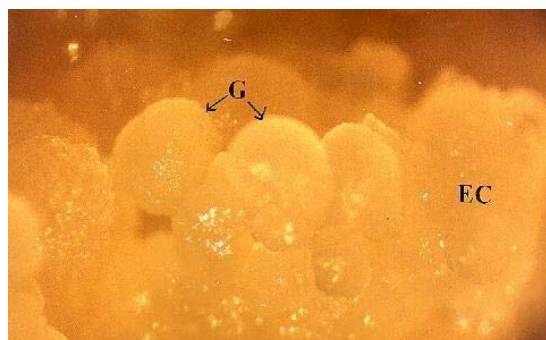


Fig. 2. Globular somatic embryo (G) on the surface of garlic embryogenic calli (EC)

3.3 Somatic embryo maturation and repetitive somatic embryogenesis

In the present study, mature somatic embryo was obtained on embryo maturation medium (EMM) containing $0.01 \mu\text{M}$ 2,4-D and combination of sucrose and sorbitol as carbon source. After 2-3 weeks on the EMM, globular embryo developed into mature embryo (Fig. 3). It was shown that reducing the concentration of 2,4-D in the medium resulted in further growth and development of embryo. *Zea mays* embryoid did not develop into late globular or coleoptilar stage in the presence of 2,4-D, but reducing or eliminating 2,4-D caused further growth and development of the embryo¹¹. Therefore, 2,4-D is required only for callus induction or competent single embryogenic cells induction¹³.

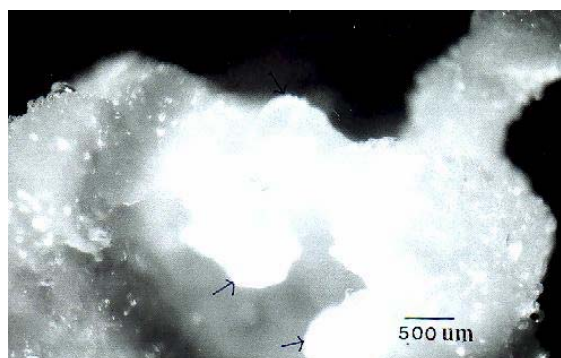


Fig. 3. Mature somatic embryo (arrow) of garlic on maturation medium 2-3 weeks of culture

Mature somatic embryos were then subcultured into repetitive somatic embryogenesis medium (RSEM), a MS medium without plant growth regulator. On the RSEM, repetitive somatic embryogenesis occurred, shown by secondary somatic embryo formation from the first somatic embryo (Fig. 4 a and b). Repetitive somatic

embryogenesis in Oak (*Quercus* spp) occurred due to the presence of auxin¹⁴. In the present study, the embryo was subcultured into the medium without plant growth regulator. Therefore, it was assumed that endogenous auxin has a role in the somatic embryogenesis.

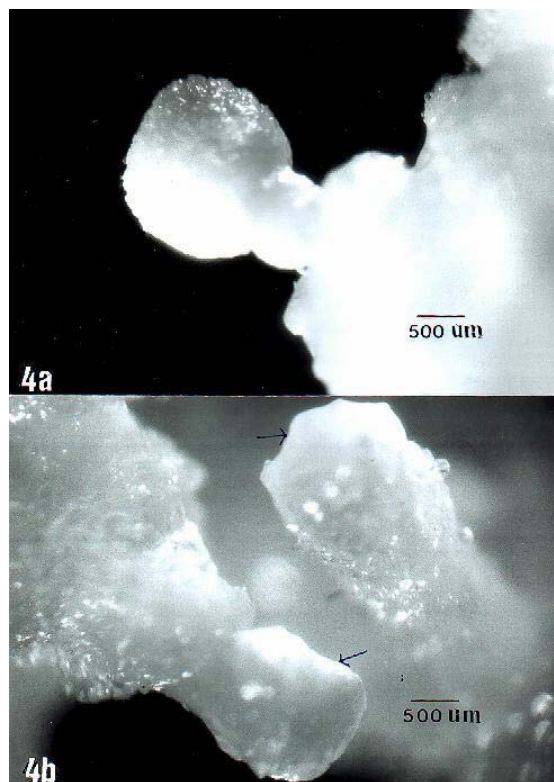


Fig. 4a and b. Repetitive somatic embryo (arrow) of garlic on repetitive somatic embryogenesis medium, a. globular somatic embryo, b. mature somatic embryo

3.4 SEM observation

Observation of somatic embryogenesis using dissecting microscope gave information focused on morphological change of the embryo, whereas by using SEM, surface structural change could be revealed. The observation using SEM was conducted on embryogenic callus aged 5-7 weeks of culture on ECIM (Embryogenic callus induction medium). Proembryo structure and somatic embryo in early transition stage were found on 5 weeks of culture. Each embryo structure has its own character on cell wall surface. The surface of proembryo cells was smooth (Fig. 5). The smooth surface structure was also shown in proembryo of rice⁸). Whereas in the early transition stage of somatic embryo (Fig. 6a), the projections that were connected with cellulose microfibril were seen on its surface (Fig.6b). At 7 weeks of culture, the embryo was on late transition stage (Fig. 7a). Its surface was covered by fibrillar material (Fig. 7b.).

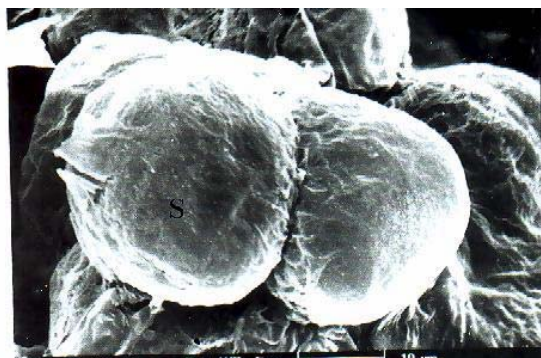


Fig. 5. Scanning electron micrograph of proembryo cells, S= smooth surface

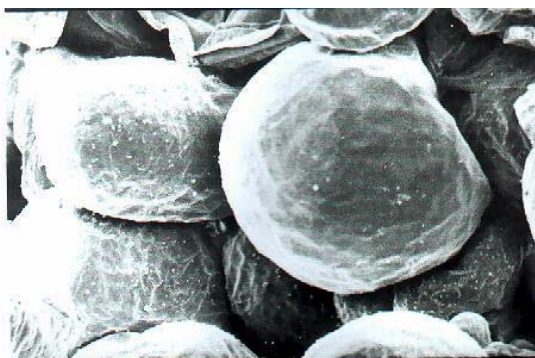


Fig. 6a. Scanning electron micrograph of early transition stage somatic embryo of garlic.

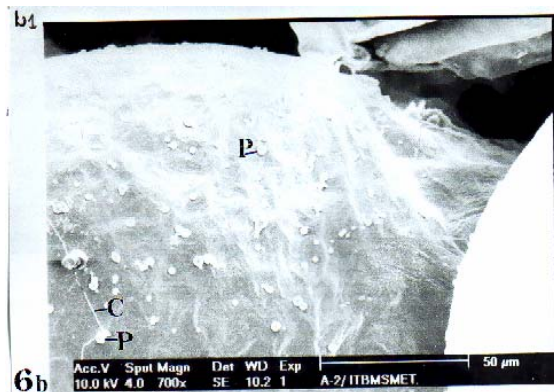


Fig. 6b. Scanning electron micrograph of surface structure of early transition stage garlic somatic embryo. P= Projection, C= Cellulose microfibril



Fig. 7a. Scanning electron micrograph of late transition stage somatic embryo of garlic.

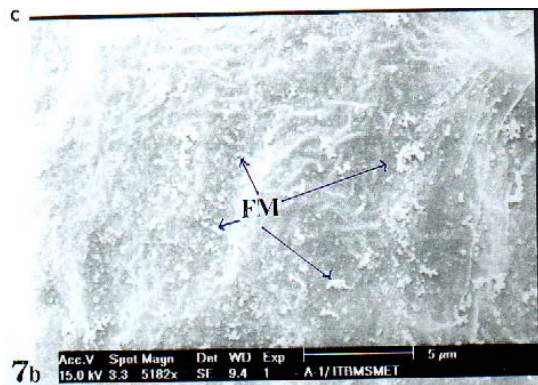


Fig. 7b. Scanning electron micrograph of surface structure of late transition stage garlic somatic embryo. FM = Fibrillar material

When the transition stage of embryo developed into globular stage, the entire surface of the embryo has been covered by a new cell wall (Fig. 8a and b) Fig. 8a shows globular embryo with suspensor (unicellular origin). It means that the embryo was derived from a single cell. Therefore, it will reduce somaclonal variation. This advantage will become important when we applied the somatic embryo in plant genetic transformation¹⁵. Fig. 8b shows surface structure of globular embryo that has been covered by the new cell wall.

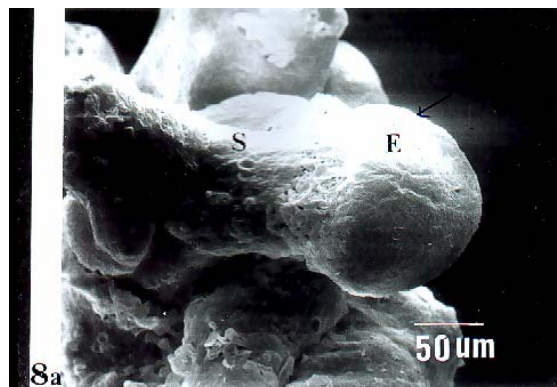


Fig. 8a . Scanning electron micrograph of garlic globular somatic embryo (arrow) E= Embryo, S=Suspensor

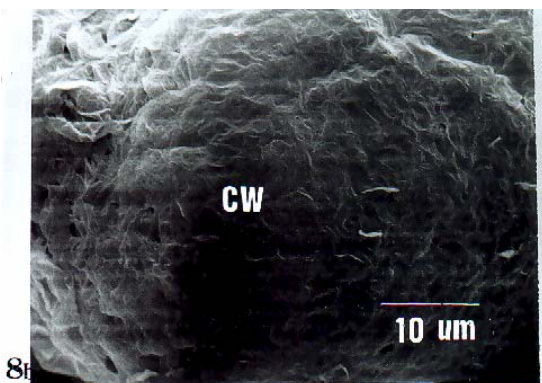


Fig. 8b. Scanning electron micrograph of surface structure of garlic globular somatic embryo, CW = Cell wall

Surface structural change from smooth to new cell wall formation shows that the cell wall was plastis¹⁶⁾. It was proven in the previous research in rice somatic embryogenesis⁸⁾. The cell wall differentiation was marked by breakdown and cell wall recovery¹⁷⁾. In the present study, the cell wall differentiation was shown by the formation of new cellulose microfibril.

In 2 weeks of culture on maturation medium, the embryo has matured (Fig 9a). The surface structure did not undergo many changes or has been stable (Fig.9b), but the embryo elongated. In addition, single cotyledon stage of embryo was also found (Fig. 10). Its surface has been stable (no more breakdown and cell wall recovery) because the matured and single cotyledon embryos were one new individual.



Fig. 9a. Scanning electron micrograph of garlic mature somatic embryo



Fig. 9b. Scanning electron micrograph of surface structure of garlic mature somatic embryo



Fig. 10. Scanning electron micrograph of single cotyledon somatic embryo of garlic

4. Conclusion

1. The optimum concentration of 2,4-D for inducing embryogenic callus from the root tip explant of *Allium sativum* L. cv. Lumbu Hijau was 0.1 μM . Reducing the concentration of 2,4-D to 0.01 μM resulted in the formation of mature embryo and single cotyledon. On the medium without plant growth regulator the repetitive somatic embryogenesis occurred.
2. Stages of somatic embryogenesis in *Allium sativum* L. cv. Lumbu Hijau were as follow: proembryo, transition embryo, globular embryo, mature embryo and single cotyledon
3. Surface structure of the embryo had a typical characteristic on every stage of somatic embryo development. Therefore, it could be used to determine the stages of embryogenesis

Acknowledgement

We thank Dr. Rizkita Rachmi Esyanti (Institut Teknologi Bandung, Indonesia) for proofreading the manuscript, Dra. Ida Nurwendah (Sekolah Tinggi Farmasi, Bandung, Indonesia) for doing tissue culture, Mr. Ahmadi (Institut Teknologi Bandung, Indonesia) for assisting in SEM observation, for this research.

References

1. Fujiwara M, & Natata T. "Induction of tumour immunity with tumour cells treated with extract of garlic (*Allium sativum*)", *Nature* **216**, 83 (1967).
2. Wibowo, S., "Budidaya Bawang", Penebar Swadaya. Jakarta, 1989.
3. Swadana, R., "Budidaya Bawang Putih", Penerbit Kanisius. Yogyakarta. p. 11-25 (1995).
4. Hartmann, T.H , Kester, E.D , Davies, T.F., & Geneve, L.R. "Plant Propagation : Principles and Practices", Sixth edition. Prentice Hall. New York 1997.
5. Haque M.S., Wada T., & Hattori K., "Efficient Plant Regeneration in Garlic through Somatic Embryogenesis from Root Tip Explants", *Plant Prod. Sci.* **1** (3), 216 (1998).
6. Abe, T. & Futsuhara Y.. "Efficient Plant Regeneration by Somatic Embryogenesis from Root Callus Tissue of Rice (*Oryza sativa* L.)", *J.Plant Physiol.*, **121**, 111 (1985).
7. Ozawa, K., & Komamine A., "Establishment of System of High Frequency Embryogenesis from Long-term Suspension Culture of Rice (*Oryza sativa* L.)", *Theor. Appl. Genet.*, **77**, 205 (1989).
8. Mariani, T.S., Miyake H., & Takeoka Y. "Changes in Surface Structure during Direct Somatic Embryogenesis in Rice Scutellum Observed by Scanning Electron Microscopy", *Plant Prod. Sci.*, **1** (3), 223 (1998).
9. Murashige, T., & Skoog, F., "A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures", *Plant Physiol.*, **15**, 437 (1962).

10. Vasil, V., Vasil I.K., & Chin-yi Lu., "Somatic Embryogenesis in Long-term Callus Cultures of *Zea Mays* L.(Gramineae)", *Amer. J. Bot.* , **71(1)**, 158 (1984).
11. Fransz, P.F., "Cytodifferentiation during Callus Initiation and Somatic Embryogenesis in *Zea mays* L.", p.73-113 (1988).
12. Abe, T. & Futsuhara Y., "Selection of Higher Regenerative Callus and Change in Isozyme Pattern In Rice (*Oryza sativa* L). ", *Theor.Appl. Genet.*, **78**, 648 (1989).
13. Fukuda, H., Ito, M., Sugiyama, M., & Komamine, A., "Mechanism of The Proliferation & Differentiation of Plant Cells in Cell culture System", *Int.J.Dev. Biology.* **38**, 287 (1994).
14. Wilhelm, E. "Somatic Embryogenesis in Oak (*Quercus spp.*)". *In Vitro Cell. Dev. Biol.-Plant* **36**, 349 (2000).
15. Williams, E.G., & Maheswaran G., "Somatic Embryogenesis : Factor Influencing Coordinated Behaviour of Cells as Embryogenic Group", *Ann. Bot.* **57**, 433 (1986).
16. Mariani, T.S., "Studies on Direct Embryogenesis in Rice (*Oryza sativa* L.)", Dissertation, Nagoya University, Japan, (1999).
17. Burgess, J. "An Introduction to Plant Cell Development", Cambridge University Press. Cambridge. 67-92 (1985).