

Effect of Cell Density and *Benzyl Amino Purine* on the Growth of Somatic Embryo of Citrus Mandarin Batu 55 (*Citrus reticulata* Blanco.) in Liquid Culture

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Abstract

Citrus mandarin Batu 55 (*Citrus reticulata* Blanco.) is one of Indonesian fruits commodities that have high economic value and consumers demand. The propagation of citrus mandarin by plant tissue culture generally was carried out on solid medium. The liquid culture system could increase cell multiplication therefore it became alternative method of plant propagation through somatic embryogenesis. The effect of initial cell density and Benzyl Amino Purin (BAP) concentration in liquid media were investigated. The initial cells density and right concentration of BAP given in media can increase cell proliferation of somatic embryo in liquid culture. Globular somatic embryo were cultured on Murashige and Tucker media with initial cell density 4, 6, 8 and 10 mgL⁻¹ and BAP 0, 0.25, 0.5, and 0.75 mgL⁻¹. Growth evaluation of somatic embryo were obtained by weighing fresh and dry weight every 2 weeks for 8 weeks for initial cell density treatment and 6 weeks of BAP treatment. The result of the research showed that cell density affect the growth of somatic embryo of citrus mandarin. Somatic embryo with low cell density showed slower growth compared than high cell density. Peak growth occurred in 6th cultured with cell density 10 mgL⁻¹. In addition to cell density, the growth of somatic embryo in liquid culture was affected by BAP. The growth of somatic embryo on the media containing BAP showed better results than without BAP. The highest BAP concentration on media showed fresh and dry weight of somatic embryo increased. In this research, growth of somatic embryo is not optimal yet because fresh and dry weights of somatic embryo still increase with high concentration 0.75 mgL⁻¹ of BAP.

Keywords: Benzyl Amino Purine, cell density, citrus mandarin, liquid medium, somatic embryo.

INTRODUCTION

Citrus mandarin (*Citrus reticulata* B.) is one of Indonesian fruit commodities that has high economic value and consumers demand [1]. Citrus mandarin high vitamin and sweet taste [2]. But, some problems faced in cultivation of citrus mandarin found are limited land provision, low seedlings available, and a difficulty to get well seeds for high quality crops. Propagation of citrus mandarin has been developed by plant tissue technology through somatic embryogenesis. Generally, this technique using solid medium which has several disadvantage that are the low absorption of nutrients and easily accumulated toxic compounds [3].

Culture in liquid medium has several benefits including high multiplication of cells, the entire cell surface is in direct contact with the medium, better aeration, and there is no gradient nutrients and gas in the medium [4]. Liquid

culture system have the potency for bioreactor culture, alternative method of micropropagation of citrus mandarin [5].

Initial cell density and growth regulators on media are important factors that affect the growth and development of cells in liquid medium [7]. Cell density during early inoculation effects the growth of somatic embryo in liquid culture [8]. In general, low cell density causes low proliferation, but high cell density inhibit cell growth because of accelerate accumulation of toxic compound in liquid culture, resulted of an imbalance between medium and cell density in the medium [8]. The highest multiplication of somatic embryo of *Citrus suhuinensis* and *Citrus kalamondin* were obtained at cell density 4 - 6 mgL⁻¹ and 2 - 6 mgL⁻¹, respectively [2].

Benzyl Amino Purine (BAP) is one of the cytokinin compounds to induce and stimulate the growth of citrus somatic embryos. The previous report showed that 1.5 mgL⁻¹ BAP increased multiplication of somatic embryo of *C. aurantifolia* on solid media whereas 0.1 mgL⁻¹ BAP increased the multiplication of somatic embryo *C. suhuinensis* [4]. The objective of the research was to

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study the effect of initial cell density and BAP on the growth of somatic embryo in liquid culture.

MATERIALS AND METHODS

Induction and Multiplication of Somatic Embryo

Somatic embryo were induced from nucellus explants cultured on MT (Murashige and Tucker) medium +50 gL⁻¹ sucrose +3 mgL⁻¹ BAP +50 mgL⁻¹ malt extract. The culture was incubated at room temperature 24°C for two months [8]. Formed somatic embryo was subcultured on MT medium + 30 gL⁻¹ sucrose every 6 weeks for 2-3 times.

Treatment of cell density and BAP concentration

Globuler stage of somatic embryo were subcultured on liquid media MT + 30 gL⁻¹ sucrose with treated cell density of 4, 6, 8, and 10 mgL⁻¹. The cell density that resulted best growth of somatic embryo is used as basis for further experiment treatment.

BAP treatment was done with the globular stage somatic embryo subculture (best cell density) in liquid media MT + 30 gL⁻¹ sucrose + 0, 0.25, 0.5, and 0.75 mgL⁻¹ BAP. Culture was incubated on temperature 24°C and homogenated by using shaker on 230 rpm. Growth was evaluated by weighing fresh and dry weight of somatic embryo every 2 weeks for 8 weeks for cell density treatment and 6 weeks for BAP treatment.

Data Analysis

This study used a randomized complete block design with repetition as a group. Factors used were initial cell density and BAP concentration. Data were analyzed using ANOVA and Duncan advanced test using significance $\alpha < 0.05$.

RESULT AND DISCUSSION

Somatic embryo in liquid culture showed a friable texture and white color (Fig. 1). Initial cell

density of somatic embryo affected cell growth and proliferation of somatic embryo in liquid culture. The growth of somatic embryo at high initial cell density was better than low initial cell density (Fig. 1).

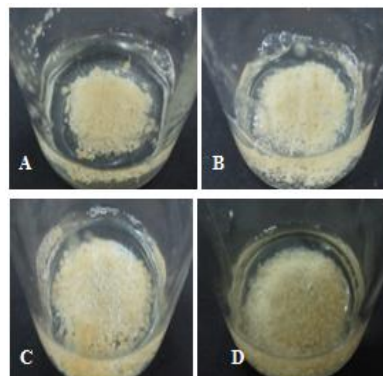


Figure 1. The growth of somatic embryo with different initial cell density at 6 weeks of culture in liquid media; A. 4 mgL⁻¹; B. 6 mgL⁻¹; C. 8 mgL⁻¹; D. 10 mgL⁻¹

The inoculation of culture with different initial cell density affected fresh and dry weight of somatic embryo in liquid culture. The growth of somatic embryo at low initial cell density was slow, while at high initial cell density it was faster. The optimum fresh weight of somatic embryo was at 6 weeks of culture and decreased after 6 weeks of culture (Fig. 2A), but the dry weight of somatic embryo still continue to increase until 8 weeks culture (Fig.2B).

The multiplication of somatic embryo with 10 mgL⁻¹ initial cell density was higher than the others, whereas the culture with 4 mgL⁻¹ cell density result the lowest multiplication of somatic embryo. Fresh and dry weights of somatic embryo in culture with initial cell density 10 mgL⁻¹ were 2.93 g and 0.15 g.

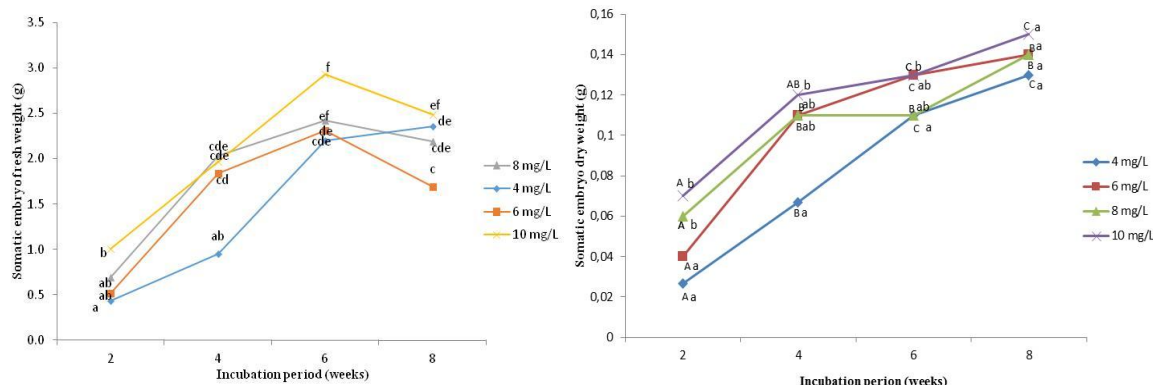


Figure 2. Fresh and dry weight of somatic embryo for 8 weeks in liquid culture; A. Fresh weight, B. Dry weight; The same letter for cell density and incubation period showed no significantly different by Duncan test $\alpha < 0.05$.

Conversely, fresh and dry weight of somatic embryo in culture with cell density 4 mgL^{-1} was only 2.20 g and 0.11 g (Fig. 2). The optimum multiplication of somatic embryos was obtained 6 weeks incubation period and initial cell density 10 mgL^{-1} .

Beside cell density, BAP also increased the growth of somatic embryo in liquid culture. The growth of somatic embryos in liquid medium with the addition of BAP was better than without BAP (Fig. 3).

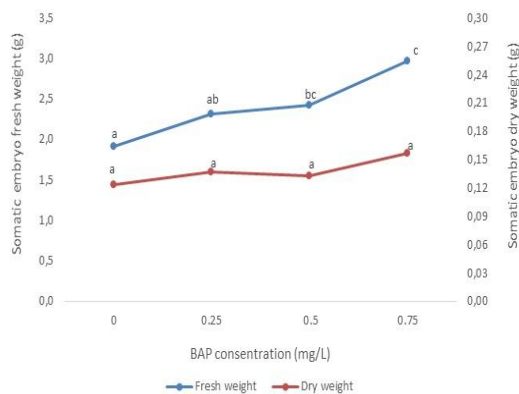


Figure 3. Fresh and dry weight of somatic embryo in liquid culture without BAP in the 6 weeks; The same letter for fresh and dry weight showed no significantly different by Duncan test $\alpha < 0.05$

The growth of somatic embryo on media which added with various concentrations of BAP was observed by measuring the fresh and dry weight at 6 weeks cultures. The growth of somatic embryo on medium with the addition of BAP resulted better than without BAP. The higher concentration of BAP on media, the increasing of somatic embryo the optimum growth was higher. Fresh and dry weight of somatic embryo in liquid medium containing 0.75 mgL^{-1} BAP were 2.97 g and 0.17 g. Otherwise, fresh and dry weight of somatic embryo on media without BAP were only 2.01 g and 0.11 g. However, the optimum growth of somatic embryo in liquid media containing BAP was not obtained yet, because of the growth of somatic embryos on the highest of BAP was given in this research was still increase.

The initial cell density of culture was an important factor of growth needed to obtain maximum growth of cells in liquid medium. Culture with high cell density accelerate the proliferation and vice versa. High cell density absorbed nutrients faster in liquid medium than lower cell density. However, when the number of

cells of somatic embryo in liquid medium had already maximum, the cell proliferation should automatically inhibited, because an imbalance between media and nutrients for cell in the medium [7]. The decrease of cell proliferation might also caused by the use of prolonged liquid media which accumulated toxic compound [8].

Previous study reported that prolonged incubation period caused the color changes of media and became finally decreased cell proliferation. Incubation period was also determined cell growth of somatic embryo in liquid media [2]. Culturing somatic embryo for too long period in liquid culture caused disturbance of osmotic process within the cell, therefore the water content of somatic embryo became very low. It became one of the factors that decrease proliferation of somatic embryo in liquid culture [10].

The growth regulators BAP in the culture media stimulated cell proliferation in some species. A range different BAP concentration has been used to induce somatic and embryogenesis shoot regeneration of citrus and *Chrysanthemum* [11]. Multiplication of somatic embryos in *Chrysanthemum* showed the best growth at media containing supplement of 1.0 mgL^{-1} BAP [11], whereas in *C. aurantifolia* and *C. sinensis* at media with concentration of 1.5 and 2 mgL^{-1} BAP. The higher concentrations of BAP in liquid culture had a negative effect on embryogenic cell growth. The addition of 3 mgL^{-1} BAP into liquid media decreased the growth of somatic embryo in *C. suhuinensis* [2]. Liquid culture system significantly accelerated multiplication of somatic embryo, thus it has a promising potential as micro-propagation technique in citrus through somatic embryogenesis.

CONCLUSION

The optimum growth of somatic embryo in citrus mandarin were obtained at initial cell density of 10 mgL^{-1} and 6th incubation period culture. Addition of plant growth regulator BAP on media increased multiplication of *Citrus* somatic embryo in liquid culture.

REFERENCES

[1] Hardiyanto, A. Supriyanto. 2010. Jeruk mandarin variety Batu 55. Indonesian Citrus and Subtropical Fruit Research Institute. Malang.

- [2] Agrisimanto, D., N.M. Noor, Ibrahim R., A. Mohamad. 2010. Efficient somatic embryo production of Limau Madu (*Citrus suhuiensis* Hort.Ex Tanaka. *African J. Biotech.* 11. 2879-2888.
- [3] Al Taha, H.A.K., A.M. Jasim, M.F. Abbas. 2012. Somatic embryogenesis and plantlet regeneration from nucleus tissues of local orange (*Citrus sinensis* (L.) Osbeck). *Acta Agr.* 99 (2). 185-189.
- [4] Devy, Y., Hardiyanto. 2012. Perbanyakkan massal embrio kalamondin melalui teknologi somatik embriogenesis melalui bioreaktor. *Jurnal Holtikultura.* 22(1). 1-7.
- [5] Kobayashi, T., K. Higashi, H. Kamada. 2001. Stimulatory and inhibitory conditioning factors that regulate cell proliferation and morphogenesis in plant cell culture. *Rev. Plant Biotech.* 18(2). 93-99.
- [6] Vantu, S. 2008. Somatic embryogenesis in *Rubus caesius* L. suspension cultures. *J. Biol. Veg.* 2 (2). 104-108.
- [7] Mukhtar, R., M.M. Khan, R. Rafiq, A. Shahid, F.A. Khan. 2005. *In Vitro* regeneration and somatic embryogenesis in *Citrus aurantifolia* and *Citrus sinensis*. *Int. J. Agr. Biol.* 7(3). 518-520.
- [8] Moiseeva, N.A., Y.N. Serebryakova, L. Nardi, S. Lucretti, Butekno R.G. 2006. Organization of initial stages of somatic embryogenesis in tissue culture of *Citrus Sinensis* cv. Taracco at the organism level. *Russ. J. Plant Physiol.* 53. 548-555.
- [9] Widoretno, W., C. Martasari, F.F. Nirmala. 2013. Pengaruh sukrosa dan fotoperiode terhadap embriogenesis Jeruk Mandarin Batu 55 (*Citrus reticulata Blanco*). *J. Hort. Indonesia.* 4. 44-53.
- [10] Umehara, M., S. Ogita, H. Sasamoto, H. Kamada. 2004. Inhibitory Factors of somatic embryogenesis regulated suspension culture of Japanese Larch (*Larix leptolepis* Gordon). *Plant Biotech.* 21 (2). 87-94.
- [11] Mani, T., K. Senthil. 2011. Multiplication of *Chrysanthemum* through somatic embryogenesis. *Asian J. Pharm. Tech.* 1 (1). 13-16.