

## Effects of Low Temperature on Somatic Embryos Growth, Maturation and Plantlet Regeneration of Citrus Mandarin var Batu 55 (*Citrus reticulata* Blanco.)

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### Abstract

This study was aimed to determine the effect of incubation at 4°C temperature during multiplication stage of somatic embryos on growth, maturation and plantlets regeneration of citrus Mandarin var Batu 55. Globular somatic embryos were cultured on MT (Murashige & Tucker) medium and incubated at 4°C temperature for 0, 2, 4, 6, 8 weeks. Maturation of somatic embryos was induced by transferring globular somatic embryos on maturation medium (MT + 50 mg l<sup>-1</sup> malt extract + 73 mM sorbitol + 73 mM galactose). Cotyledonary embryo was regenerated on MT medium + 50 mg l<sup>-1</sup> malt extract + 30 g l<sup>-1</sup> sucrose + 2 ppm GA<sub>3</sub>. The research showed that maturation of somatic embryos at 4°C temperature inhibited growth and maturation of somatic embryos. Fresh weight of somatic embryo incubated at 4°C for 2 weeks lower than fresh weight of embryo cultured without incubation at 4°C, and continuous decline in longer incubation period. Maturation percentage of embryos without incubation at 4°C temperature was 15%, but embryos incubated at 4°C temperature were lower than 9%. There was no effect of incubation at 4°C temperature during somatic embryos multiplication stage on plantlet regeneration percentage.

**Keywords:** *Citrus reticulata*, maturation, plantlet regeneration, somatic embryo

### INTRODUCTION

Citrus Mandarin var Batu 55 is one of the most economically important citrus variety in Indonesia caused of sweet taste a bit sour, soft textured fruit, easy to peel and almost seedless. This superior characteristic caused high market demand. But this productivity could not been able to fulfill the national citrus demand. One of the efforts to increase productivity was through the provision of citrus seedling. Micropropagation through somatic embryogenesis is an effective method of plant propagation in large scale for mass seedling production such as citrus [1], apple [2], mango [3] and other fruit plant.

Regeneration of citrus Mandarin var Batu 55 through culture techniques have been successfully carried through somatic embryogenesis techniques using nucellar as an explant [4]. However plant regenerated from in vitro technique has long juvenile period ranging from 3-5 years [5]. Thus it was capable for producing citrus rootstock. Therefore it was necessary to find a method to produce citrus scion with short juvenile period.

Modification of culture conditions such as photoperiod and low temperature treatment has been able to reduce the juvenile period of plants seedling regenerated from in vitro culture.

Low temperature exposure successfully stimulated flowering in some plants such as in *Cinchorium intybus* [6] and *Oenothera fruticosa* [7]. Low temperature treatment given during somatic embryos multiplication stage could produce plant with short juvenile period, but it was also affected the growth and development of somatic embryos. This study aimed to evaluate the effect of low temperatures treatment on somatic embryos growth, maturation and plantlets regeneration of citrus Mandarin var Batu 55.

### MATERIALS AND METHODS

#### Induction and multiplication of somatic embryos

Somatic embryos were obtained from nucellus explant cultured on MT medium +50 g l<sup>-1</sup> sucrose + 3 ppm BAP + 50 mg l<sup>-1</sup> malt extract, cultured incubated at room temperature as long as two months [8]. Embryogenic calli subcultured on MT medium + 30 g l<sup>-1</sup> sucrose every 6 weeks, and cultured at 25°C ± 2°C. Somatic embryos that have undergone three times subculture used as explants for low temperature treatment.

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### Low temperature treatment

Globular stage somatic embryos were incubated at 4°C for 2, 4, 6, 8 weeks, whereas the control was embryos incubated in temperature room. Each treatment was repeated 5 times (5 bottles), each bottle was cultured with one clump embryos (0.1 g). After each incubation period, embryos were incubated in room temperature as long as two months. Somatic embryo growth was examined by measuring somatic embryo fresh weight.

### Somatic embryo maturation and plantlet regeneration

Embryo without incubation at 4°C and embryos in temperature 4°C treatment were transferred to MT + 50 mgL<sup>-1</sup> malt extract + 73 mM sorbitol + 73 mM galactose. Culture incubated in room temperature for three months, then number of cotyledonary embryo and maturation percentage was counted.

Cotyledonary somatic embryos were germinated on MT medium + 30 gL<sup>-1</sup> sucrose + 50 mgL<sup>-1</sup> malt extract + 2 ppm GA<sub>3</sub>. Each bottle was cultured with 10 embryos and incubated at room temperature for three months, then number of plantlet and plantlet regeneration percentage were counted.

### Data Analysis

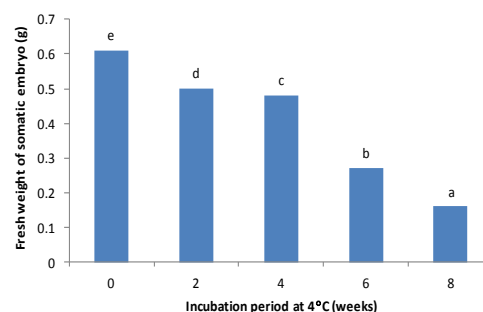
The data of somatic embryo growth, percentage of maturation and percentage of plantlet regeneration were analyzed by the ANOVA test ( $p < 0.05$ ). Significant difference was tested by Duncan's multiple range test ( $p < 0.05$ ).

## RESULT AND DISCUSSION

### Effect of low temperature treatment on somatic embryo growth

Low temperature did not affect the morphology of embryos, but affected somatic embryo growth reflected by different size of embryo clump in each incubation period. Increasing incubation period at 4°C increasingly inhibited the growth of somatic embryos, it is shown in smaller clump size. Incubation at 4°C temperature during embryo multiplication stage decreased somatic embryo fresh weight. Fresh weight of somatic embryo incubated at 4°C for 2 weeks was lower than embryos incubated at room temperature and continuous decline in longer incubation period. Fresh weight of untreated embryo reaches 0.61 g, treated embryos are lower than 0.5 g. The lowest fresh weight was shown by somatic embryo cultured

in 4 °C treatment for 8 weeks in the amount of 0.17 g (Fig. 1).

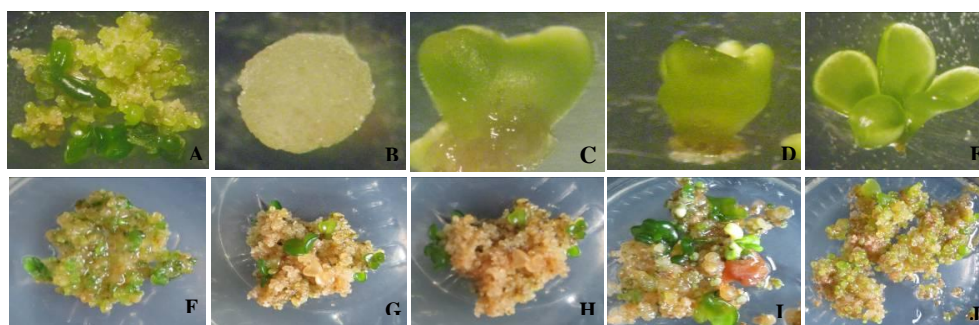


**Figure 1.** The effect of low temperature treatment on fresh weight of somatic embryo  
The same letter for each incubation period showed no significant difference between means by Duncan test 5%.

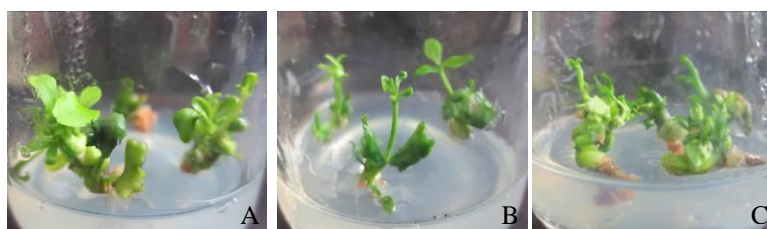
### Effect of low temperature treatment on somatic embryo maturation and plantlet regeneration

After transferred on maturation medium as long as two months embryos were mature, shown by changing in color of embryos, which initially appears milky white to yellow with green spots. After three months cultured on maturation medium, there were found globular embryo (Fig. 2 A-E). Incubation at 4°C during multiplication stage inhibited somatic embryo growth and maturation. Somatic embryos incubated at 4°C produce fewer cotyledonary embryos than embryos incubated at room temperature (Fig. 2F-J). Somatic embryos incubated at room temperature and embryos incubated at 4°C able to regenerate into plantlets (Figure 3A-B). Plantlets which regenerated from embryos incubated at 4°C more than 4 weeks showed stunted growth (Figure 3C).

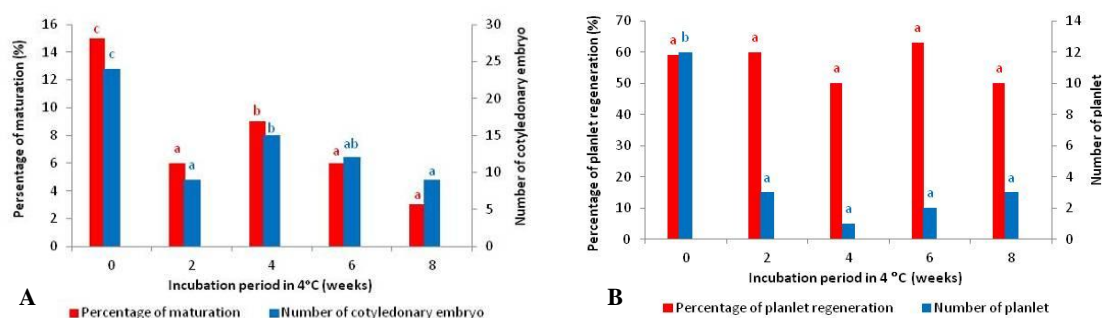
Incubation of somatic embryos at 4°C decreased maturation percentage. Somatic embryos incubated at 4°C show a higher percentage of maturation and number of cotyledonary embryos. The number of cotyledonary embryo and maturation percentage decreased with increasing incubation period at 4°C treatment. Maturation percentage of embryos incubated at room temperature was 15% with number of cotyledonary embryos was 25 embryos, whereas embryos incubated at 4°C for 4 weeks was 9% with number of cotyledonary embryo was 15. Maturation percentage and number of cotyledonary embryo in longer incubation period were less than 6% with number of cotyledonary embryo was about 5-11 embryos (Fig. 4A).



**Figure 2.** Developmental stage of *Citrus Mandarin* var *Batu 55* somatic embryos after incubated at 4 °C temperature. A. Embryo started mature; B. Globular phase; C. Heart-shaped; D. Torpedo; E. Cotyledonary; F. Embryos incubated at room temperature; G. Embryos incubated at 4 °C for 2 weeks; H. 4 weeks; I. 6 weeks; J. 8 weeks



**Figure 3.** Plantlets regeneration from somatic embryos of *Citrus Mandarin* var *Batu 55*. A. Planlet regenerated from embryos incubated at room temperature; B-C. Plantlet regenerated from embryos incubated at 4 °C for 2 and 8 weeks.



**Figure 4.** The percentage of somatic embryo maturation and the percentage of planlet regeneration of *Citrus Keprok Batu 55*. A. Percentage of maturation; B. Percentage of plantlet regeneration. The same letter for each maturation and plantlet regeneration percentage showed no significantly difference between means by Duncan test 5%.

There was no significant difference in planlet regeneration percentage between embryos incubated at room temperature and embryos at 4 °C. Planlet regeneration percentage of embryos incubated at room temperature and embryos incubated at 4 °C was less than 60%.

Although there was no significant difference of planlet regeneration percentage, but there was a significant difference between numbers of plantlet. Incubation of somatic embryos at 4 °C temperature decreased the number of plantlet, number of plantlet regenerated from embryos incubated at room temperature was reach 12, whereas number of plantlet regenerated from embryos incubated at 4 °C less than 3 (Fig. 4B).

Incubation at 4 °C temperature during somatic embryo multiplication step inhibited growth and

development of somatic embryos. This may occur because 4 °C temperature cause physiological stress that interfere with cell metabolism such as photosynthesis, respiration, water content, and the stability of the membrane, cell ultrastructure damage. Furthermore it led to decreased cell division and differentiation and embryogenesis [9]. Low temperature treatment causes changes in wall and cell membrane interactions, which cause any changes in gene expression and proteins synthesis that play a role in the development of somatic embryos. In addition, these proteins may be a key protein that induces maturation and germination of somatic embryos resulted an abnormal embryos [10]. Abnormal embryos morphology tends to be slow to form plantlets because it can lead to blocking the

development of the embryo in the early stages of somatic embryo development. Plantlets formed from abnormal embryos showed gigantic growth followed by none formation of buds, but capable of forming roots [11].

Eventhough low temperature caused abnormalities of somatic embryos, low temperature was also essential for maturing somatic embryo and germination in *Vitis vinifera* x *Vitis rupestris*. Globular stage embryo culture of grape (*Vitis vinifera*) was treated with 4°C temperature for two weeks, showed increasing in somatic embryo maturation percentage [12].

## CONCLUSION

Incubation at 4°C temperature affected growth and development of somatic embryo in Citrus Mandarin var Batu 55. Incubation at 4°C decreased somatic embryo fresh weight and percentage of maturation. Somatic embryo incubated at room temperature and embryos incubated at 4°C able to regenerated into planlet. Although there was no affect on plantlets regeneration percentage, but number of planlet regenerated from embryo incubated at 4°C was lower. The lowest fresh weigh, maturation and plantlets regeneration percentage showed by embryos incubated at 4°C temperature treatment for 8 weeks, in addition regenerated plantlets showed stunted growth.

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