

Effects of Sugar Type and Concentration on Batu 55 Mandarin (*Citrus reticulata* Blanco.) Somatic Embryo Maturation

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ABSTRACT

When obtaining plantlets, embryo maturation and simultaneous germination are important steps in plant micropropagation via somatic embryogenesis. Several studies have shown that the use of carbohydrates as a carbon source plays a significant role in inducing somatic embryo development in certain plant species. This study aimed to establish a somatic embryo maturation protocol for Batu 55 Mandarin (*C. reticulata* Blanco.) by examining the effect of various types of sugar and concentrations. The results showed that the type and concentration of sugar added to the medium affect the somatic embryo maturation of Batu 55 Mandarin. Galactose and maltose enhance somatic embryo maturation more effectively than does sorbitol. The combination of galactose or maltose with sorbitol was able to improve somatic embryo maturation more effectively than galactose or maltose alone. The combination of galactose and sorbitol enhanced the maturation of somatic embryos more effectively than did change the concentrations of maltose or sorbitol. It can be concluded that sugar type and concentration had effects on citrus somatic embryo development. The combination of sorbitol (36.5 mM) with galactose 73 mM was able to augment citrus somatic embryo maturation more effectively than the other concentrations applied.

Keywords: *Citrus reticulata*; galactose; maturation; somatic embryo; sorbitol

INTRODUCTION

Somatic embryogenesis was commonly used method for citrus micropropagation in vitro. Several studies concluded success on induction and proliferation of citrus somatic embryo. Induction of somatic embryo maturation difficulty is

one of the main inhibition for successful somatic embryogenesis on citrus and several other plant species, such as soybean (Walker & Parrott, 2001), Pinus (Li, Huang, Murphy, & Gbur, 1998) and Picea (Misra, Attree, Leal, & Fowke, 1993; Bozhkov & von Arnold, 1998). Carbohydrate was commonly used as carbon source for tissue development on in-vitro culture (Iraqi & Tremblay, 2001). This compound was considered significant for somatic embryo maturation on several species.

Not only as a source of nutrition, but also as osmoticum (Li, Huang, Murphy, & Gbur, 1998) that affected both angiosperm (Canhoto & Cruz, 1994; Blanc, Michaux-Ferrière, Teisson, Lardet, & Carron, 1999) and gymnosperm embryogenesis. Carbon source and concentration had an evident effect on maturation and germination of the somatic embryo on some plant species (Nasim et al., 2010; Corredoira, Ballester, & Vieitez, 2003; Körbes & Droste, 2005). Maltose as a carbon source and osmoticum was effective in somatic embryo development of *Allium sativum* (Nasim et al., 2010), *Castanea sativa* Mill. (Corredoira, Ballester, & Vieitez, 2003) and *Pinus nigra* (Salajova, Salaj, & Kormutak, 1999). While the additional concentration of sucrose in the medium was able to elevate somatic embryo development on *Dianthus caryophyllus* L. (Karami, Deljou, & Pour, 2008), *Brassica napus* (Slesak & Przywara, 2003) and *Glycine max* L. (Körbes & Droste, 2005). Sucrose combined with gellan gum encouraged the recovery of the somatic embryo in cotyledon phase (Ramarosandratana, Harvengt, Bouvet, Calvayrac, & Pâques, 2001). Increasing the sucrose concentration or glucose substitution with mannitol or sorbitol also affected the maturation of *Pinus strobus* somatic embryos (Garin et al. 2000). The purpose of this study is to develop maturation protocol for the somatic embryo of Batu 55 Mandarin by examining effects

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of various sugar type and concentration. Batu 55 Mandarin is a local citrus variety that has several features, such as greenish yellow to yellow color, sweet and fresh taste, soft textured flesh, high content of vitamin C and high productivity made it popular on farmer and consumer.

MATERIALS AND METHODS

Somatic Embryo Induction and Multiplication

Embryogenic callus was induced from nucellus explant of Batu 55 Mandarin collected from young fruits (80-90 days after anthesis) taken from the mother tree. Explant surfaces were sterilized by submerging fruits in 96% alcohol for 10 seconds and 20% sodium hypochlorite for 20 minutes. Nucellus explant was isolated from fruit and cultured in MT medium (Murashige & Tucker, 1969) supplemented with 146 mM sucrose, 500 mgL⁻¹ malt extract, and 8gL⁻¹ agar. Formed embryogenic callus was transferred into new embryogenesis medium. The culture was incubated at 25±2°C in the dark condition.

Somatic Embryo Maturation by Various Sugar Type and Concentration Treatment

Maturation of somatic embryo was induced by culturing globular somatic embryo on maturation medium for cotyledonary embryo development. Maturation medium contained MT medium supplemented with 500 mgL⁻¹ malt extract and galactose (73 mM) or maltose (73 mM) combined with sorbitol at several concentration (0, 36.5, 73, 109.5 and 146 mM). The media was solidified with 8gL⁻¹ agar. The cultures grew in

these media for 8 weeks and were incubated in the bright area at 25 ± 2 °C temperature. The experiments were done in three replications; each of them was cultured one clump of the somatic embryo (0.1g). After 8 weeks, somatic embryo growth was examined by weighing somatic embryo fresh weight, while somatic embryo maturation was measured by counting each development phase of the embryo (globular, heart, torpedo, and cotyledon). The data of somatic embryo fresh weight and development phase were analyzed using ANOVA with α 5%. When a significant difference was found between treatments, analysis continued to BNT advance test.

RESULTS AND DISCUSSION

Results

The development stages of the citrus somatic embryo were consecutively globular, heart, torpedo, and cotyledon phase (Figure 1). Citrus somatic embryo at globular phase had round shape, yellowish white color, and soft structure. Bilateral symmetry is apparent from heart-shaped somatic embryos. At this stage, pro-vascular cells also started to differentiate and embryo color turned greenish yellow and slightly harder structure. At torpedo phase, embryo grew lengthwise and laterally, while the cotyledonary stage was indicated by the emergence of cotyledon. Embryo at torpedo and cotyledon phase had slightly greener color (Figure 1).

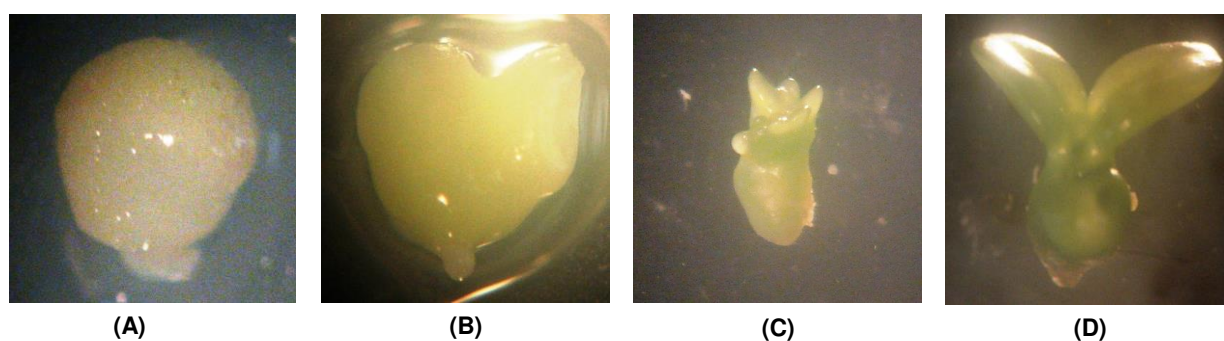


Figure 1. The developmental stage of Batu 55 Mandarin somatic embryo: (A). Globular; (B). Heart; (C). Torpedo and (D). Cotyledon.

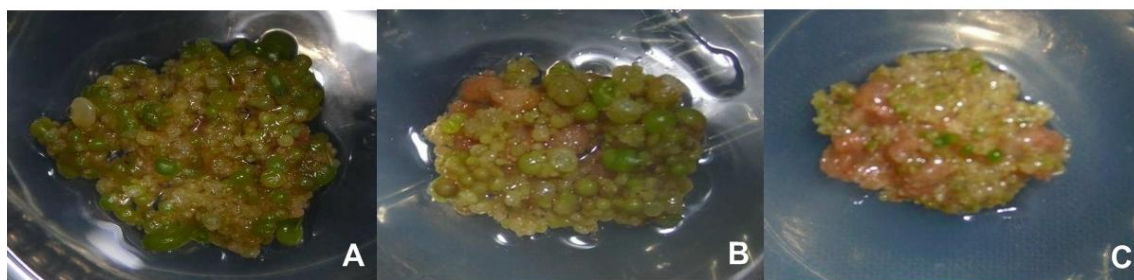


Figure 2. Growth and development of somatic embryo cultured on medium supplemented different kind of sugar: (A) Galactose; (B) Maltose and (C) Sorbitol

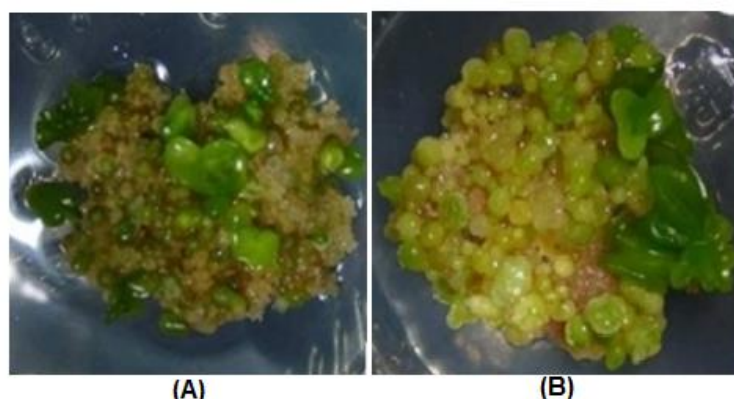


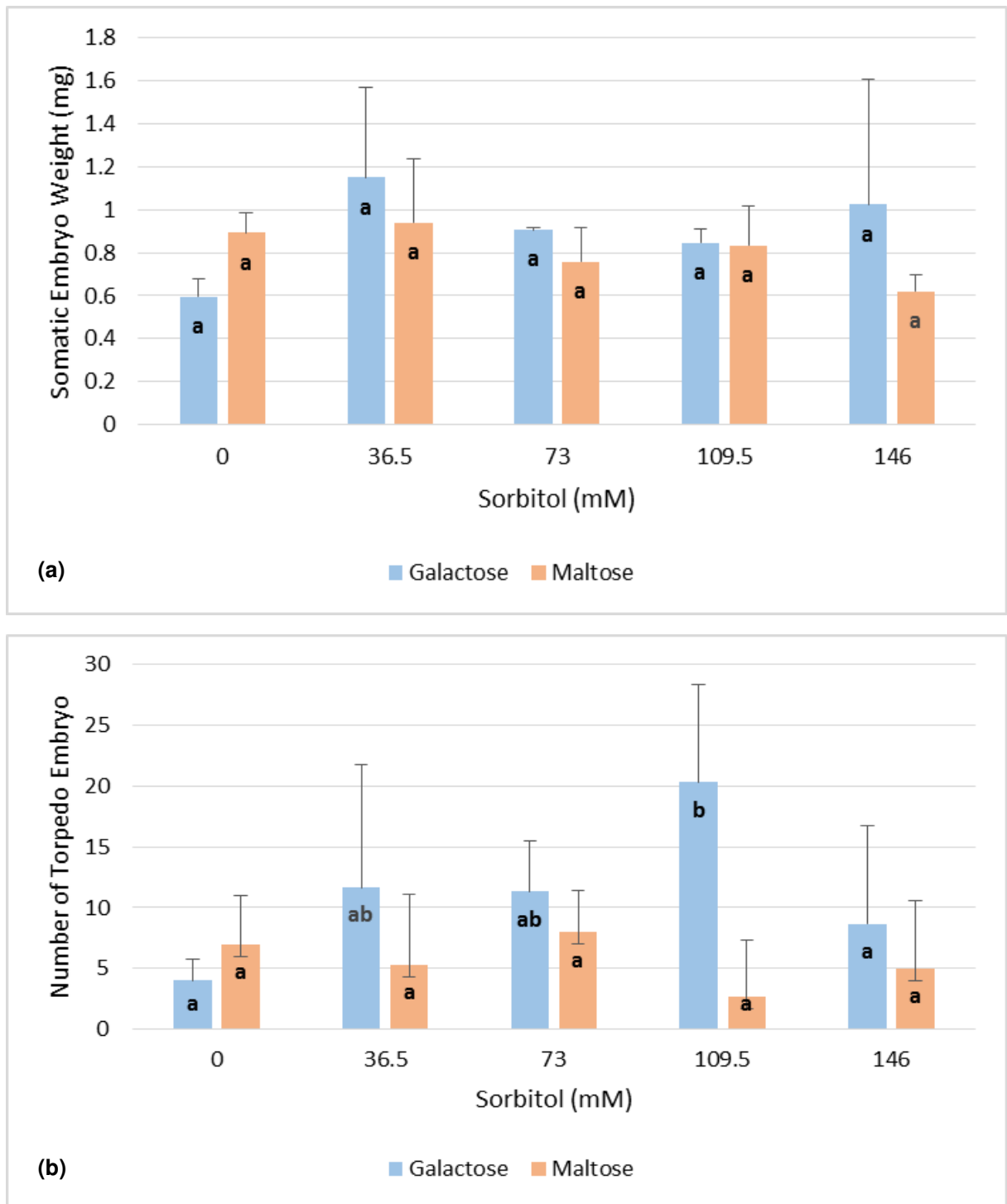
Figure 3. Growth and development of the citrus somatic embryo in maturation medium containing galactose or maltose combined with sorbitol at a different concentration; Galactose (A) Maltose (B)

Sugar type affected the growth and development of the citrus somatic embryo. Among the three types of sugar used, galactose was more effective than maltose and sorbitol in enhancing the growth and development of somatic embryos. In a medium with additional sorbitol, somatic embryos growth was not quite good and some of them had a reddish color. Besides of better growth, somatic embryos in medium with additional galactose or maltose also more mature compared to the sorbitol treatment, indicated with yellowish green or green coloring (Figure 2).

Total somatic embryo and globular embryo numbers in medium with galactose were higher than those in maltose or sorbitol treatment. Nevertheless, somatic embryo development in the maltose-supplemented medium was superior to that observed in medium with galactose. This was shown by the amounts of the embryo at heart, torpedo and cotyledon phase on medium with maltose were higher than those in galactose. Growth and development of somatic embryos in medium with additional sorbitol were poor. It is fewer numbers of formed somatic embryos, its

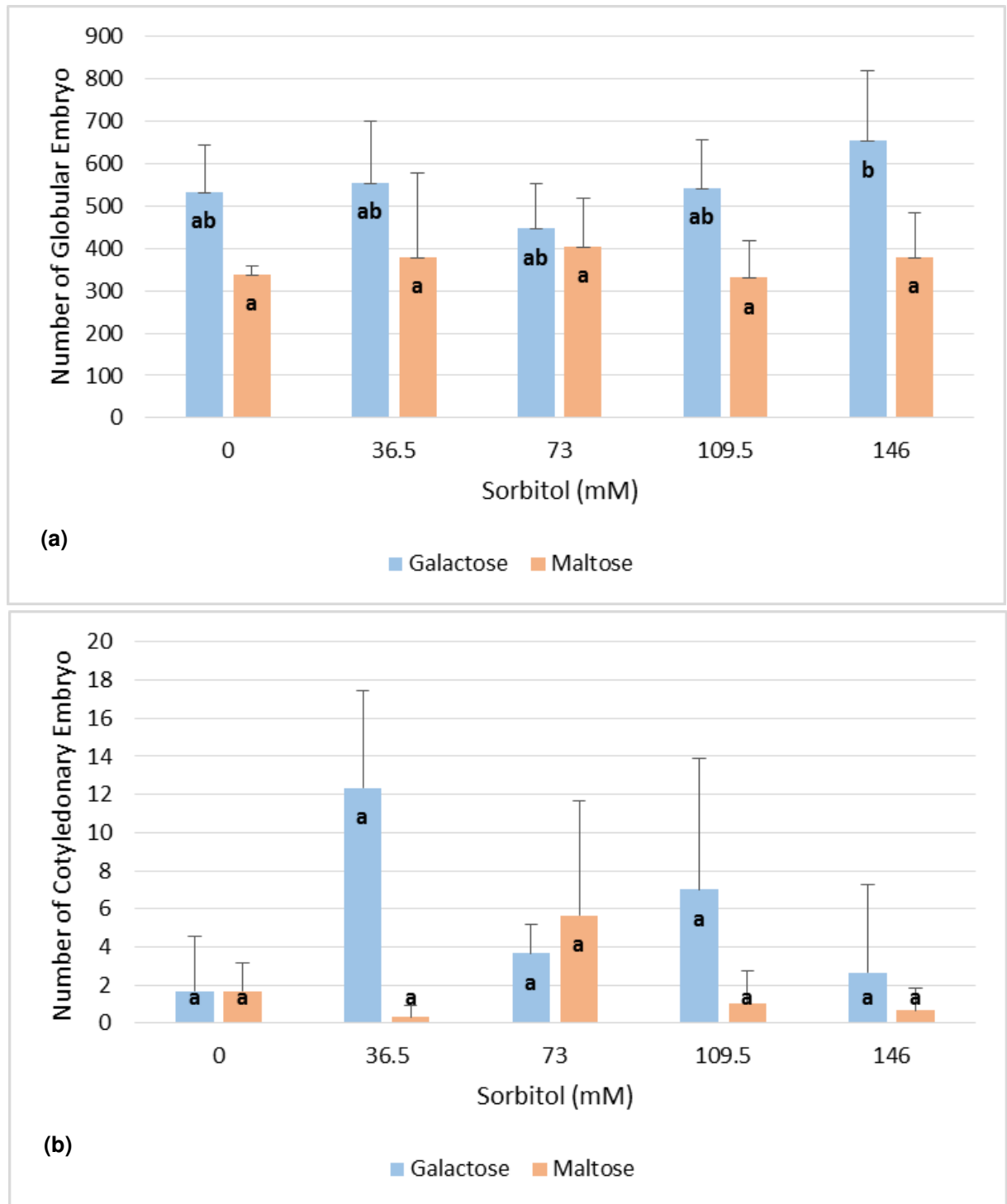
amount at heart and torpedo phase was also smaller. In a medium with sorbitol, the cotyledonary embryo was not found.

Although additional galactose or maltose in the medium was able to improve embryo development more effectively than sucrose, nevertheless the application of one type of sugar had not able to elevate somatic embryo maturation up to a maximum point. Hence, for maximizing the development of the citrus somatic embryo, two types of sugars combination were needed; by combining galactose or maltose with sorbitol. Somatic embryo growth and development were enhanced in culture medium supplemented with two types of sugar such as galactose and sorbitol or maltose and sorbitol, in comparison to medium containing a single type of sugar. On medium containing galactose or maltose combined with sorbitol, many embryos were developed towards more advanced stage i.e.: heart, torpedo, and cotyledon phases which were shown by green color and certain shape according to each of embryo development phase (Figure 3).



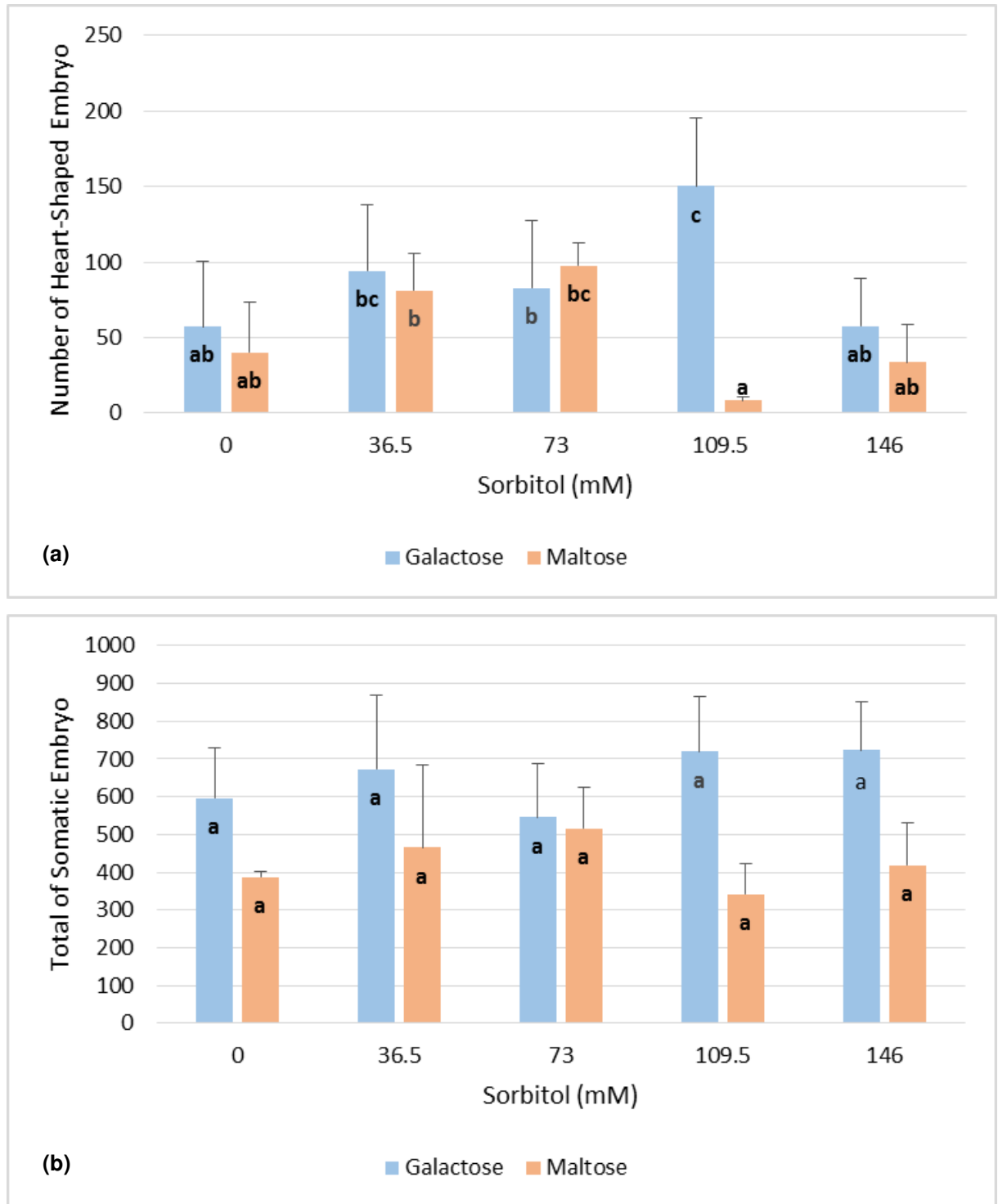
Remarks: The same alphabet at the same variable indicates insignificant difference based on Duncan test at $\alpha=5\%$

Figure 4. The effect of various sorbitol concentrations combined with either galactose or maltose on growth and maturation of citrus somatic embryo: (a) Somatic embryo weight (mg); (b) A number of torpedo embryo



Remarks: The same alphabet at the same variable indicates insignificant difference based on Duncan test at $\alpha=5\%$

Figure 5. The effect of various sorbitol concentrations combined with either galactose or maltose on growth and maturation of citrus somatic embryo: (a) A number of the globular embryo; (b) A number of the cotyledonary embryo



Remarks: The same alphabet at the same variable indicates insignificant difference based on Duncan test at $\alpha=5\%$

Figure 6. The effect of various sorbitol concentrations combined with either galactose or maltose on growth and maturation of citrus somatic embryo; (a) A number of the heart-shaped embryo; (b) Total somatic embryo

Additional sorbitol combined with galactose or maltose in the medium was able to increase the formation and development of the somatic embryo. Somatic embryo growth and development were enhanced in medium containing galactose and sorbitol as compared to medium with maltose and sorbitol. The higher sorbitol concentration applied with galactose (36.5, 73, 109.5, 146 mM), the more formed somatic embryo. But if sorbitol was combined with maltose, the increasing numbers of formed somatic embryo were limited to 73 mM sorbitol concentration. Higher sorbitol concentration combined with maltose started to inhibit the formation of the somatic embryo (Figure 4, Figure 5 and Figure 6).

In addition, to enhance embryo growth, additional sorbitol combined with galactose or maltose also enhanced somatic embryo maturation. Galactose and sorbitol combination improved somatic embryo maturation than maltose and sorbitol. Mediums containing galactose and sorbitol combination were able to form 58-150 heart-shaped embryos, 13-20 embryos at torpedo phase, and 3-12 cotyledonary embryos. While mediums containing maltose and sorbitol combination were only able to produce 34-98 heart-shaped embryos, 3-8 torpedo stages embryos, and 1-6 cotyledonary somatic embryos. Sorbitol 73 mM combined with galactose increasing the somatic embryo maturation higher than other sorbitol concentration. The numbers of the somatic embryo at torpedo and cotyledonary phase at this concentration was 17 and 12, while in medium containing galactose only was 4 and 2 respectively. Whereas the combination of 73 mM sorbitol with maltose resulted in the best development of somatic embryo compared to the rest combination. Sorbitol 73 mM combined with maltose had 8 embryos at torpedo phase and 6 cotyledonary embryos.

DISCUSSIONS

Plant somatic embryogenesis was a process consisted of several stages that are: induction, proliferation, maturation and germination (Weber, Borisjuk, & Wobus, 1997). Each stage required specific culture condition which was including nutrient medium composition. Embryo maturation and simultaneous germination to obtain plantlet were one of the many important stages on in-vitro micropropagation via somatic embryogenesis.

Maturation steps were needed for the development of the mature somatic embryo, after proliferation phase and embryogenic tissue initiation. Somatic embryo showed poor germination when it was not gone through embryo maturation phase before. On maturation phase, cell specification programs shifted to food reserve accumulation programs by adjusting embryo for post-embryonic development (Yadegari & Goldberg, 1997). During maturation, some processes were needed for embryo formation, such as synthesis and accumulation of food reserve, germination suppression, desiccation tolerance acquisition and dormancy induction at several species (Koornneef & Karssen, 1994). Several components in culture medium have been known to stimulate somatic embryo maturation. Sugar played an important role in somatic embryo development in some plants (Nasim *et al.*, 2010, Salaj, Matúšová, & Salaj, 2004). In some species, embryo development could be controlled by changing sugar content in the medium (Körbes & Droste, 2005; Nasim *et al.*, 2010). During maturation, carbohydrate supply was an important factor which influenced the quality and number of the embryo. Carbohydrate added into culture medium are used as carbon source, osmotic and also contribute in histodifferentiation of the somatic embryo by means of directing gene expression regulation (Lipavská *et al.* 2000). Carbon source and concentration had a significant effect on the growth and development of the somatic embryo. Galactose and maltose had a better effect than sorbitol on citrus somatic embryo development.

Nevertheless, a combination of sorbitol with galactose and maltose was able to enhance the citrus somatic embryo maturation. The effect of carbohydrate type and concentration in culture medium on improving somatic embryo maturation had also been observed in some plant species. Maltose has been found can both act as carbon source and effective osmotic in somatic embryo development of *Allium sativum* (Nasim *et al.*, 2010), *Pinus kesiya* (Choudhury, Kumaria, & Tandon, 2008), *Glycine max* (Körbes & Droste, 2005), *Castanea sativa* (Corredoira, Ballester, & Vieitez, 2003) and *Abies nordmanniana* (Noorgard, 1997). The increasing of somatic embryo maturation *Pinus kesiya* in medium containing maltose was caused by reducing water availability in medium (Choudhury, Kumaria, & Tandon, 2008), whereas, maltose was able to

increase the development of alfalfa somatic embryo because of the maltose effects was primarily of nutritional and not of osmotic nature (Strickland, Nichol, McCall, & Stuart, 1987). According to Noorgard (1997), maltose improved the somatic embryo maturation of *Abies nordmanniana* by reducing hydrolysis. Low hydrolysis of maltose was a biochemical signal for somatic embryo formation, in the opposite of sucrose fast hydrolysis which caused increased of hexose content and reserve compounds in the case of cell proliferation rate (Blanc, Lardet, Martin, Jacob, & Carron, 2002). Low absorption ability and slow maltose metabolism could also induce nutrition stress, causing elevation of somatic embryo maturation on *Hevea brasiliensis* (Blanc, Michaux-Ferrière, Teisson, Lardet, & Carron, 1999). Several authors confirmed that maltose with ABA capable in activating general metabolic pathway (Finkelstein and Gibson 2001; Leo and Shee 2003). The addition of maltose in culture medium was a base for morphologic reorganization and histodifferentiation of embryogenic cells toward development stages (Steiner, do Nascimento Vieira, Maldonado, & Guerra, 2005). Sucrose substitution with lactose or galactose at proliferation stage of conifer somatic embryo was useful in decreasing disorganized growth and directing a production of the immature embryo to a better organization.

These effects seemed to be more evident compared to maltose. If embryos were going to be transferred from lactose/galactose treatment into maturation medium, they had greater tendency to develop into mature embryo stage compared to the one cultured in medium supplemented with a different sugar, such as maltose. Thus, a higher mature embryo with greater uniformity and better quality could be formed to produce more vigorous plantlets and plants. Growth and development inhibition of somatic embryo in maturation medium which only contained sorbitol and elevation of somatic embryo maturation in medium with combined sorbitol either with galactose or mannitol indicated that sorbitol merely could not support proliferation and maturation of citrus somatic embryo. According to Tholakalabavi, Zwiazek, & Thorpe (1994), sorbitol and mannitol absorbed and slowly metabolized by the plants. Sorbitol was ineffective for maturation and germination of *Pistacia vera* embryo (Canhoto & Cruz, 1994) and inducing necrosis in feijoa somatic embryo (Canhoto &

Cruz, 1994). Although the medium contained mannitol or sorbitol, it was ineffective for the maturation of oak somatic embryos. Nevertheless, a combination of these compounds with sucrose was able to stimulate embryo maturation better than the use of sucrose alone. It was caused by the disability of oak cells to utilize mannitol or sorbitol as an energy source and or structural carbon. Sorbitol considered to be inactive at some plants, such as at some Fagaceae member because the transported carbon source was sucrose, but in some other plants, such as several Rosaceae and Oleaceae member, it played in carbohydrate metabolism (Zimmermann & Ziegler, 1975). Sorbitol was useful for apple micropropagation (Pua & Chong, 1984) and corn somatic embryogenesis. Sorbitol was translocated effectively in apple cells and transformed into glucose by sorbitol oxidase or into fructose by sorbitol dehydrogenase. The effectiveness of sorbitol in apple micropropagation and corn somatic embryogenesis has also been contributed to the high level of those sorbitol metabolic enzymes. Sorbitol ineffectiveness on citrus somatic embryo development was likely due to citrus cells inability to utilize sorbitol as an energy source or the absence of enzymes capable turning sorbitol into glucose or fructose in the citrus tissue.

CONCLUSION

Sugar type and concentration had effects on citrus somatic embryo development. Galactose and maltose enhanced embryo maturation more effectively than did sorbitol. The combination of two sugar types could stimulate somatic embryo development more effectively than the use of a single sugar type. The combination of galactose and sorbitol augmented the maturation of somatic embryos in comparison to the effects observed with a combination of maltose and sorbitol. The combination of sorbitol 36.5 mM combined with galactose 73 mM was able to enhance citrus somatic embryo maturation more effectively than the other concentrations applied.

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Wahyu Widoretno *et al*: *Effects of Sugar Type and Concentration on Batu 55 Mandarin*.....

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