INTRODUCTION

*Citrus reticulata* Blanco cv. Madu is one of the most popular citrus mandarin species in South-East Asia. However, the production is limited due to the common citrus disease, greening, caused by Liberobacter (Bendix & Lewis, 2018). Hence, vegetative propagation of infected plant using traditional method is strongly avoided to reduce the spread of the disease (Siverio et al., 2017). Furthermore, the conventional propagation was slow to facilitate mass production of seedlings (Singh, Singh, Nongalleima, Moirangthem, & Devi, 2013).

A promising alternative way to propagate citrus seedlings to produce disease free plants and thus through tissue culture (Beom, Ho, Man, Hoon, & Yun, 2017). This clonal propagation or micropropagation allows to produce an offspring totally similar to the mother plant (El-Sherif, 2019) through organogenesis and/or embryogenesis pathway (Chiancone & Germanà, 2013).

Recently embryogenesis on citrus have been successfully conducted on citrus rootstock (Chiancone & Germanà, 2013), *Citrus limon* and *C. sinensis* (Meziane et al., 2017), *Citrus reticulata* Blanco (Widoretno, Indriyani, Martasari, & Hakin, 2017), and *Citrus nobilis* (Nurwahyuni & Sinaga, 2018). While micropropagation of citrus in bioreactor has been recently reported on *Citrus × latifolia* (Yu. Tanaka) (Bulbarela-Marini et al., 2019). However, till date, rapid cell proliferation and mass somatic embryo induction using a bioreactor has not been reported for *Citrus reticulata* cv. Madu.

In a bioreactor, environmental factors, both chemical and physical, are completely regulated for optimum growth of cells and/or organs (Cui et al., 2014; Thanh, Murthy, & Paek, 2014). Those factors should be determined prior application of bioreactor for high yield production (Nartop, 2018). Bioreactors

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ABSTRACT

Embryogenic cell (EC) growth and proliferation as well as somatic embryo induction were examined in a bioreactor culture using Murashige and Skoog basal medium particularly for the influence of 6-benzyladenine (BA) concentration, initial cell density and aeration rate. Embryogenic callus was induced from seeds of *Citrus reticulata* Blanco cv. Madu. The cell suspension in a 3-L bioreactor exhibited maximum cell growth following the addition of 1.5 mg/l of BA. The fresh weight (FW) of the cells after 28 days of growth was found to increase from an initial of 5.5 g cell culture to 57.3 g, a 10.4-fold increase and the maximum growth rate (GR) of the cells (0.33 g/day), again by the 7th day of culture. In the cell density experiment, ECs at a concentration of 5.5 g/l constituted the most effective inoculum, reaching the highest GR of ECs (0.52 g/day), again by the 7th day of culture. In the aeration experiment, the highest EC GR of 2.6 g/day was obtained at the maximum aeration rate of 1.5 vvm (air volume medium/ volume/min). After 28 days of somatic embryogenesis, 79% of ECs became somatic embryos, of which 29% were at cotyledonary stage.
are increasingly used for rapid and mass production of somatic embryos for several reasons. The bioreactor is beneficial for the development of mass regeneration systems that are healthy organs and the growth of valuable plants throughout the year (Cui et al., 2014). Different type of bioreactors were confirmed as one of the most efficient tissue culture methods to enhance mass production of desired plant cells or organs from cells, organs, or whole plants (Eibl et al., 2018; Suman, 2017) with high secondary metabolite content (Eibl et al., 2018; Werner, Maschke, Eibl, & Eibl, 2018). The bioreactor provides automation system (Egertsdotter, Ahmad, & Clapham, 2019) for scaling-up plant cells and tissue at industrial level (Nartop, 2018). A typical air-lift bioreactor is a vessel that has several ports for explant inoculation, for aeration (inlet and outlet) and for medium injection (Paek, Chakrabarty, & Hahn, 2005). An air-lift bioreactor of the type used in this study has been used in the pilot production of thousands of embryos of ginseng, grape and orchid (Cui et al., 2014; Park, Ahn, Lee, Murthy, & Paek, 2005; Shohael, Murthy, & Paek, 2014; Sun et al., 2016; Tapia et al., 2009).

The rate of cell proliferation can be increased by manipulation of plant growth regulators and carbohydrate sources in a culture medium, thereby dramatically improving the rate of regeneration (Phillips & Garda, 2019; Satdive, Fulzele, & Eapen, 2007). Although the addition of a cytokinin, such as 6-benzyladenine (BA) and kinetin, is considered essential for micropropagation (Phillips & Garda, 2019) such as embryogenesis, what is required for optimal regeneration varies by genotype (Carimi & Pasquale, 2003). Furthermore, initial cell density (ICD) (Sun et al., 2016) and aeration rate (Özcana, Sargin, & Göksungurb, 2014) are important for cell or organ growth in a bioreactor. Cell density has been found to be one of the key factors for achieving maximum GR in air-lift bioreactor cultures (Gorret et al., 2004). According to Sinlaparaya, Duanghaklang, and Panichajakul (2007), there exists a critical ICD for better growth of cells in the liquid medium producing fresh cell biomass. They observed that cell culture needs a minimum number of cells in the medium at the time of inoculation, which determines the level of nutrition required for each cell for initial cell division. The stimulation of cell growth with increased ICD might result from cell–cell communication among the inoculum cells (Akalezi, Liu, Li, Yu, & Zhong, 1999). The increase in ICD has been shown to achieve higher levels of cell proliferation in citrus (Februyani, Widoretno, & Indriyani, 2016) and even more in bioreactor as reported in ginseng (Thanh et al., 2014).

Therefore, this study aimed to develop a protocol for rapid and extensive proliferation of C. reticulata cv. Madu embryogenic cells (ECs) by optimizing BA concentration, ICD and aeration rate; and subsequent somatic embryo conversion by optimizing aeration rate in an air-lift bioreactor system.

**MATERIALS AND METHODS**

The studies were conducted in tissue culture laboratory of Nuclear Malaysia and Indonesian Citrus and Subtropical Fruits Research Institute (ICSTFRI). The proembryonic masses were first initiated in 2011 and refreshed in 2015. Developments of small scale of bioreactors were conducted in ICISFRI in 2016 while bigger scale in nuclear Malaysia.

**Culture Establishment**

Immature citrus fruits (80 - 120 days after anthesis) of Citrus reticulata Blanco cv. Madu were harvested from mother plant. Fruits were washed under running tap water and surface sterilization using 70% ethanol for 15 minutes and 20% chlorox for 10 minutes respectively. Fruits were washed by autoclaved distilled water prior seed extraction. Those seeds along the segment wall adjacent to fruit axis were carefully excised under aseptic conditions in a laminar air flow cabinet, and were directly cultured on MS basal medium (Duchefa M220) supplemented with 0.5 g/l malt extract, 50 g/l sucrose, 0.27% (w/v) gelrite and 3 mg/l BA to induce embryogenic calli. The pH of the medium was adjusted to 5.7 ± 0.1 with 0.5 M KOH before autoclaving at 121 °C and 15 psi (103 kPa) for 15 min. The embryogenic calli were subcultured biweekly on to the fresh medium. EC suspension culture was initiated following Agisimanto, Normah, Ibrahim, and Mohamad (2012) and was then maintained with biweekly by deflasking with 50% reduction of BA concentration.

**EC Proliferation in An Air-Lift Bioreactor Culture**

The cultures were grown in 3-l air-lift bulb-type bubble bioreactors (Fig. 1). Three individual experiments were carried out sequentially to test the effects on cell proliferation. The first experiment was BA concentration (1.25, 1.50, and 1.75 mg/l), the
second was ICD (4.5, 5.0, and 5.5 g/l) and the third experiment was aeration rate (0.5, 1.0, and 1.5 vvm). All the cultures were incubated at 25 ± 2 ºC under continuous illumination with cool white fluorescent lamps with light intensity of 35 µmol/m²/s. Air was pumped into the bioreactor via a 0.22 µm filter. A flow meter placed between the air filter and pump on each culture to control the rate of aeration. The cells were harvested every week for kinetic growth measurements. To measure the fresh weight (FW) of the biomass and the dry weight (DW), cells were dried on filter paper for 2 h and then dried at 50 - 60 ºC for 6 h prior to measuring fresh weight and for 48 h prior to measuring dry weight. The growth rate (GR) of the cells was calculated as:

$$GR = \frac{[HFW (g) - IFW (g)]}{PC (t)}$$

Remarks: GR : Growth rate; HFW : Harvested Fresh Weight; IFW : Inoculated fresh weight; PC : Period of culture

Aeration Rate Effect on Somatic Embryogenesis in An Air-Lift Bioreactor

During subsequent somatic embryogenesis, the effects of aeration rates (0.5, 1.0, and 1.5 vvm) were again studied. ECs at 100 g were inoculated into a 3 l air-lift bioreactor containing 2 l MS medium supplemented with 0.5 g/l malt extract, 110 mM sorbitol and 36 mM galactose following Agisimanto et al. (2012). For conversion, the embryos were subsequently transferred to semisolid MS medium containing 0.5 g/l of ME, 88 mM sucrose, and 10 g/l agar. The cultures were maintained as condition mentioned above. The numbers of globular-shaped, heart-shaped, torpedo-shaped, and cotyledonary-stage embryos formed in each treatment were identified visually using a stereomicroscope.

Experimental Design and Statistical Analysis

The experiments followed a completely randomized design and were carried out in triplicate. SAS and Microsoft Excel software were used for determination of analysis of variance (ANOVA) of each experiment. For means comparison, Duncan Multiple Range Test (DMRT) was employed. Probabilities less than or equal to 0.05 were considered as significant.

RESULTS AND DISCUSSION

EC Proliferation in An Air-Lift Bioreactor Culture

Addition of BA, ICD and aeration rate on the culture positively affected cell proliferation (Table 1). Addition of BA at concentrations from 1.25 to 1.50 mg/l increased the FW (Fig. 2A) from the initial weight of 5.5 g of inoculum cells to 42.7 and 57.3 g, respectively. However, addition of BA at a concentration of 1.75 mg/l resulted in FW of 48.8 g, exerting a less beneficial effect on cell growth than did BA addition at the 1.50 mg/l, yet better than that at the 1.25 mg/l. A similar pattern of growth was noted with regard to the DW (Fig. 2B). With respect to the GR (Fig. 2C), at the best concentration of BA (1.50 mg/l), the cells reached the highest rate of proliferation (GR of 0.33 g/day) on day 7 of the culture period; however, with addition of BA at 1.25 and 1.75 mg/l, a GR of only 0.22 and 0.27 g/day, respectively, was obtained.

In the present study, to examine cell proliferation in the bioreactor, BA was used in the range of 1.25 - 1.75 mg/l. It was found that BA concentration of 1.5 mg/l supported the greatest proliferation of ECs and enabled to reach the highest GR, followed by 1.75 mg/l.
### Table 1. Cell proliferation of *Citrus reticulata* cv. Madu grown under different BA, ICD and aeration rates in an air-lift bioreactor

| Factors   | FW (g)  | DW (g)  | GR (g/day) |
|-----------|---------|---------|------------|
| BA (mg/l) | *       | *       | *          |
| 1.25      | 42.7b   | 4.75b   | 0.22b      |
| 1.50      | 57.3a   | 6.41a   | 0.33a      |
| 1.75      | 48.9ab  | 5.53ab  | 0.27ab     |
| ICD (g/l) | **      | **      | **         |
| 4.5       | 82.3a   | 8.6a    | 0.34a      |
| 5.0       | 91.0b   | 9.4b    | 0.41b      |
| 5.5       | 110.2c  | 11.9c   | 0.52c      |
| AR (vvm)  | **      | **      | **         |
| 0.5       | 393.5a  | 40.7a   | 1.7a       |
| 1.0       | 506.7b  | 50.9b   | 2.2b       |
| 1.5       | 556.0b  | 56.3b   | 2.6b       |

Remarks: Means in the same column followed by the same letter are not significantly different at P ≤ 0.05 according to the Duncan Multiple Range Test.

**Fig. 2.** (A) Fresh Weight, (B) Dry Weight and (C) Growth Rate of *Citrus reticulata* cv. Madu cells grown at different concentrations of BA (1.25, 1.5 and 1.75 mg/l) in an air-lift bioreactor. Bar represented standard errors.
Cytokinin is necessary in cultured cells for the initiation of mitosis, promoting cell division and differentiation (Kieber & Schaller, 2018; Lustinec et al., 2014; Nasri et al., 2019; Takahashi & Umeda, 2014). However, higher concentrations of BA adversely affected cell proliferation in the liquid medium, as indicated by a reduced FW and DW of cells in the culture. This negative effect might due to the increase in the total amount of exogenous cytokinin deposited inside the cells, where excessive accumulation impeded cell growth. Liquid culture allowed rapid uptake of nutrient including growth regulator by cells and agitation speedy nutrient replacement at the surface of cells through diffusion (Wang, Xu, Yan, & Xu, 2019).

Increasing ICD enhanced the proliferation of cells. The addition of cells increased FW (Fig. 3A) linearly from 0 to 14 days of the culture period, with less prolific growth through the end of the culture period. The culture with the ICD of 5.5 g/l increased to 110.2 g FW at 28 days of culture, a FW higher than those obtained in the cultures with cell densities of 4.5 and 5.0 g/l, which yielded FW of 82.3 g and 91.0 g, respectively. A similar pattern of growth was noted with regard to the DW (Fig. 3B). In terms of GR, cultures with an ICD of 5.5 g/l achieved a maximum GR of 0.52 g/day on day 7, higher than the GRs of cultures with ICDs of 4.5 and 5.0 g/l (0.34 and 0.41 g/day, respectively) (Fig. 3C). All three ICDs achieved high GRs early in the culture period and maintained high GRs throughout the culture period. PEM proliferations therefore are optimal at early growing of culture in liquid. While FW increased both with optimal ICDs and with optimal aeration rates, we note that optimal aeration rates had a greater positive effect than that of optimal ICDs. Using the optimal rate of BA addition, the maximum fresh weight obtained was 57 g; with optimal ICD, fresh weight obtained was 110 g, while at the optimal aeration rate, the fresh weight was 556 g - a tremendous increase - within 28 days. Similarly even in Wang et al. (2019).

Both FW (Fig. 4A) and GR (Fig. 4B) were affected by the aeration rate. While the cells at all aeration rates grew slowly in the lag phase during the first week of culture, in the following two weeks (days 7–21) the cells grew faster. Thereafter the FW yield leveled off while GR decreased.

**Fig. 3.** (A) Fresh weight, (B) dry weight and (C) growth rate of *Citrus reticulata* cv. Madu embryogenic cells grown at different initial cell densities in a air-lift bioreactor. Bar represented standard errors.
At the end of the culture period, the aeration rate of 1.5 vvm showed the highest FW (556.0 g), while with aeration at 0.5 and 1.0 vvm, FW was 393.5 g and 506.7 g, respectively. During days 7–21, the aeration rate of 1.5 vvm supported the most profuse cell growth with GR reaching 2.6 g/day; aeration rates of 0.5 and 1.0 vvm showed in GRs of 1.7 g/day and 2.23 g/day, respectively (Fig. 4B). Cell proliferation increased in the air-lift bioreactor under an appropriate aeration rate. Aeration rate affected fluid mixing, which also depended on fluid viscosity, sedimentation, oxygen transfer, dissolved oxygen (DO), and culture growth kinetics. In the present study, it was found that in a medium with optimal nutrients and ICD, a higher proliferation of cells occurred when the aeration rate was higher. The GR on day 21 reached 2.6 g/day in the culture subjected to an aeration rate of 1.5 vvm, and considered the highest tested in this study. The improvement in GR following an increase in aeration rate might be the result of enhanced contact between the cells and medium resulting in continuous nutrient uptake and of uninterrupted forced aeration and oxygen supply. The movement of the medium that accompanied aeration ranged from moderate to excessive, depending on the aeration rate, presumably resulting in uniform nutrient distribution in the medium. These conditions resulted in better contact between the cells and medium, thus induced higher metabolism and reduced shear stress and also reduced deposition of the cells at the bottom of the bioreactor. These indicated that the developed protocol, which was initiated under lower BA, suitable ICD, and appropriate aeration rate, supported the best condition for the cells to grow and develop. Cells in liquid culture would be in full contact with the culture medium and with continuous aeration, the medium would stay in homogeneity and enable to facilitate efficient nutrient uptake (Wang et al., 2019) and continuously supplies of oxygen leading to its faster growth (Fernandes & Cabral, 2016; Nielsen, Temporiti, & Cella, 2019).

**Aeration Rate Effect on Somatic Embryogenesis in An Air-Lift Bioreactor Culture**

Higher aeration rates positively affected the differentiation of cells and formation of somatic embryos. The increase of aeration rate from 0.5 to 1.0 and to 1.5 vvm resulted in an increase in somatic embryo formation (Table 2). Higher GRs at 1.8 and 2.0 g/day at 28 days after culture were achieved from the aeration rates of 1.0 and 1.5 vvm, respectively, than that of 0.5 vvm (GR = 1.4 g/day). After 2 weeks inoculation, the percentage of globular-shaped embryos across treatments was lower than that of heart, torpedo, and cotyledon-shaped embryos (Table 3), suggesting that embryo development had already been in process for some time. While lower aeration rate allowed the conversion of globular-shaped embryos to heart- and torpedo-shaped ones, it produced significantly fewer mature or complete somatic embryos than did higher aeration rates. It was found that throughout the culture period the results of treatment with an aeration rate of 1.5 vvm were consistently superior to those of other aeration treatments (Table 3). Furthermore, at an aeration rate of 1.5 vvm, the total rate of embryo formation was 79.25% of ECs, of which 28.63% of the embryos reached cotyledonal stage by the end of the culture period, while lower aeration rates achieved lower results. A sample of somatic embryos of *Citrus reticulata* cv. Madu from our culture in the bioreactor and under the microscope was presented in Fig. 5A and B, respectively.
Table 2. Somatic embryogenesis of *Citrus reticulata* cv. Madu grown under different aeration rates in an air-lift bioreactor

| Aeration rate (vvm) | GR<sub>28</sub> (g/day) | RM<sub>28</sub> | Mixing | % SE  |
|---------------------|--------------------------|----------------|---------|-------|
| 0.5                 | 1.4<sup>c</sup>          | 70.55<sup>c</sup> | slow    | 61.25<sup>c</sup> |
| 1.0                 | 1.8<sup>b</sup>          | 91.45<sup>b</sup> | moderate| 73.38<sup>b</sup> |
| 1.5                 | 2.0<sup>a</sup>          | 101.29<sup>a</sup> | excessive| 79.25<sup>a</sup> |

Remarks: RM<sub>28</sub> = rate of multiplication at after 28 days culture, Mixing = mixing medium, %SE = Percentage of embryogenesis. Means in the same column followed by the same letter are not significantly different at P≤0.05 according to the Duncan Multiple Range Test

Table 3. Somatic embryogenesis of *Citrus reticulata* cv. Madu grown at different aeration rates in an air-lift bioreactor after 28 days in culture

| Aeration rate (vvm) | Globular     | Heart       | Torpedo     | Cotyledon   |
|---------------------|--------------|-------------|-------------|-------------|
|                     | Percentage of somatic embryos formation |                |              |             |
| 0.5                 | 10.33<sup>a</sup> | 13.99<sup>a</sup> | 17.77<sup>a</sup> | 19.16<sup>a</sup> |
| 1.0                 | 11.97<sup>a</sup> | 15.03<sup>ab</sup> | 22.22<sup>b</sup> | 24.17<sup>c</sup> |
| 1.5                 | 11.46<sup>a</sup> | 16.17<sup>b</sup> | 23.00<sup>b</sup> | 28.63<sup>c</sup> |

|                     | Number of somatic embryos |                |              |             |
|---------------------|---------------------------|----------------|-------------|-------------|
| 0.5                 | 1,182.11<sup>a</sup>    | 1,614.90<sup>a</sup> | 2,051.00<sup>a</sup> | 2,201.49<sup>a</sup> |
| 1.0                 | 1,971.90<sup>b</sup>    | 2,472.86<sup>b</sup> | 3,660.54<sup>b</sup> | 3,978.05<sup>b</sup> |

Remarks: Means in the same column followed by the same letter are not significantly different at P ≤ 0.05 according to the Duncan Multiple Range Test

Fig. 5. Somatic embryogenesis of *Citrus reticulata* cv Madu in air-lift bioreactor; (g) development of globular and cotyledonary (cot) embryos during the 3rd week of culture, (c) germinating somatic embryos, and (d) plantlet (5 weeks after germination). Bar at B, C = 1 mm, D = 1 cm
Fig. 5C and D showed the conversion process from somatic embryo at cotyledonal stage to plantlet. The rate of conversion of normal-shaped embryos was 28%, however considering the abnormally-shaped embryos might also convert to plantlets, the conversion rate might be considerably higher, as high as 50-60%. Of the plantlets emerging from normal-shaped embryos, all resembled the parental type. It was found that a higher rate of aeration clearly encouraged induction and development of somatic embryos. Inoculation with only 100 g of ECs produced 13,792 somatic embryos in various stages within just 28 days. Embryonic cells, PEM, inoculated to the culture contain differentiated embryogenically competent cells that easily converted into embryos (Efferth, 2019) in ideal condition. Of these, the most advanced cotyledonal stage was noted in 29% of the somatic embryos recovered, or approximately 79% rate of somatic embryogenesis. When embryos began to form 2 weeks after inoculation, the percentage of globular-shaped embryos across treatments was lower than that of heart, torpedo, and cotyledon-shaped embryos, indicating effective growth of cells and somatic embryo conversion as a result of higher efficient utilization and uptake of nutrition.

Somatic embryogenesis is the process to form a whole plant from a single somatic cell (El-Sherif, 2019) throughout a series of morphological and biochemical changes (Gulzar, Mujib, Rajam, Frukh, & Zafar, 2019; Nic-Can et al., 2015). Initially, regeneration of somatic tissues via somatic embryogenesis was possible in liquid cultures (Agisimanto et al., 2012; Altaf, Khan, Sadia, Jaskani, & Khan, 2017; Februyani et al., 2016; Gerolino et al., 2015; Puad, Sarji, Fathil, & Abduh, 2018), and the process was usually influenced by several factors, both in solid and liquid culture (Widoretno et al., 2017). Later, rapid protocols for large-scale cell proliferation and somatic embryogenesis of *C. sinensis* using solid medium (Kiong, Wan, Hussein, & Ibrahim, 2008) and of *C. reticulata* in flask culture (Agisimanto et al., 2012) were developed. To our knowledge, the present study is the first to report somatic embryogenesis of *C. reticulata* cv. Madu in an air-lift bioreactor. The preliminary studies to the work reported by Agisimanto et al. (2012) that used a flask culture method demonstrated that efficient somatic embryogenesis of *C. reticulata* in a liquid medium with high sucrose required low BA concentration and low ICD at the stage of cell proliferation. Consequently, further work on flask somatic embryogenesis was carried out using a medium without BA and with sucrose replaced with a combination of sorbitol and galactose (Agisimanto et al., 2012). The protocol reported here with its low but effective levels of BA supplementation, suitable ICD, and optimal aeration rate, appeared to provide an environment that was conducive for cell growth and development to somatic embryos without the stress induced by tissue culture, and was thus a promising alternative method for mass propagation of this citrus species. As somatic embryogenesis in bioreactor offered some practical advantages, especially in earlier step of in vitro phase of embryos multiplication that had opportunities to scale up for mass production. Automation process could change dramatically on cost of labor operational as reported earlier by Egertsdotter et al. (2019) on conifers.

**CONCLUSION**

A rapid system for cell proliferation and somatic embryogenesis of *Citrus reticulata* cv. Madu in an air-lift bioreactor was developed. The rates of cell proliferation were highest at BA concentration of 1.50 mg/l, ICD of 5.5 g/l, and aeration rate of 1.5 vvm, throughout the cycle of EC proliferation. At the optimal aeration rate, ECs grew profusely, exhibiting a 100-fold increase in biomass from the ICD of 5.5 g/l. Seventy nine percent of the 100 g of ECs formed somatic embryos, of which about 29% were in the advanced cotyledonal stage, on the verge of becoming plantlets. With a 28% germination rate, this study provided evidence for the potential of the air-lift bioreactor technique for production of large numbers of planting materials. This study was a first determination of the optimal levels of all three factors – initial cell density, BA addition, and aeration rate – necessary for the successful somatic embryogenesis of *Citrus reticulata* cv. Madu, suggesting the potential commercial value of this method of propagation.

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