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EFFECTS OF CARBON SOURCES, PLANT GROWTH REGULATORS AND INOCULUM SIZE ON *Citrus suhuiensis* CELL SUSPENSION CULTURE GROWTH

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ABSTRACT

Cell suspension culture of *Citrus suhuiensis* (*C. suhuiensis*) was established to determine the best growth condition based on the effects of carbon source, plant growth regulators (PGRs) and inoculum size. Friable callus from the cotyledon explant was used to initiate the cell suspension cultures. Murashige and Skoog (MS) medium supplemented with 50 g/L glucose achieved the highest cell dry weight (CDW) and μ_{max} of 23.2 g/L and 0.47 h^{-1} respectively compared to glucose at 30 g/L and other carbon sources. For PGRs effects, 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) gave the highest amount of CDW and μ_{max}; 11.91 g/L and 0.32 h^{-1}. Furthermore, the inoculum size at 10% (v/v) gave the maximum CDW and μ_{max} of 15.45 g/L and 0.71 h^{-1} respectively compared to 20% (v/v) and 30% (v/v) of inoculum. The results achieved can be used for further statistical optimization of *C. suhuiensis* cell suspension culture growth parameters in which the interactions between those parameters can be further studied and understood.

Key words: *Citrus suhuiensis,* cell suspension culture, carbon source, inoculum size, plant growth regulators (PGRs)

INTRODUCTION

Rutaceae is the family name of *Citrus suhuiensis* (*C. suhuiensis*) species, which is under Mandarin or Tangerine group of citrus classification. The local name for this fruit in Malaysia is 'Limau madu' while in other countries it is named differently such as Somkeae Wan (Thailand), Jeruk Siam (Indonesia) and Duong (Vietnam) (Elcy *et al.*, 2012). Citrus is the most important commercially cultivated fruit crops that are grown in many countries around the world and contribute to 90% or more of the total world fruit production and trade (Ladaniya, 2008). However, their plantations are often affected by nematodes, fungi, bacteria, phytoplasmas, spiroplasmas, viruses, and viroids. As examples, some diseases such as *Citrus tristeza virus* (CTV) and Citrus canker have caused reduction in the production and quality, while others have the possibility to destroy Citrus industry (Ollitrault & Navarro, 2012). Conventional genetic breeding in Citrus species presents limitations related to the reproductive biology of the genus, such as nucellar polyembryony, a long juvenile period and a high level of heterozygosity. Hence, the use of biotechnological protocols have attracted important interest to overcome these limitations (Cardoso *et al.*, 2012).

Plant tissue culture is one of the interesting methods to be used in bioproduction, bioconversion or biotransformation of the potential secondary products for large-scale production as well as biosynthetic studies (Shahzad *et al.*, 2017). In plant biology, plant cell suspension cultures are widely used as a convenient tool for invesitgating the regulation of plant growth and organized development processes, bypassing the structural

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complexity of the plant organism. The homogeneity of an *in vitro* cell population, large availability of material, high rate of cell growth and good reproducibility of conditions make cell suspension culture suitable for the analysis of complex physiological processes at the cellular and molecular levels. Moreover, plant cell cultures provide a valuable platform for the production of high-value medicinal compounds and fine chemicals (Moscatiello *et al.*, 2013). Citrus has a high content of vitamin C and other nutritional properties, and there are many studies that have been reported on this species. However, lack of research can be found on *C. suhuiensis* species especially on its suspension culture.

One of the most important factors responsible for the growth of plants *in vitro* is the composition of the culture medium. Carbohydrate or sugar is important for *in vitro* plant cell growth since they are heterotrophic which are dependent on the exogenous supply of carbon. Different carbohydrate sources have been used to support plant cell growth as well as to maintain the osmotic potential of cells. Sucrose, fructose, and glucose are the common types used as the carbon source (Sumaryono *et al.*, 2012). Besides carbon source, plant growth regulator (PGR) is also an important element in plant tissue culture since it plays vital roles in the stem elongation, tropism, and apical dominance. In tissue culture, auxins are usually used to stimulate callus production and cell growth, initiate shoots and rooting, induce somatic embryogenesis and stimulate growth from shoot apices and shoot stem culture (Saad & Elshahed, 2012). Initial inoculum density is a key factor in establishing cell suspension cultures of any plant species because cells cannot resume and even stop their active growth after the transferring and subculturing processes if the initial inoculum is below the critical cell density. All these factors are very important in the cell growth of suspension cultures, and limited information can be found on *C. suhuiensis*, specifically.

Therefore, this study aimed to establish cell suspension culture of *C. suhuiensis* by determining the best condition for the cells to grow using one-factor-at-a-time (OFAT) method. Once the cell suspension culture for *C. suhuiensis* has been well established, further investigations can be carried out for the valuable secondary metabolite production such as vitamin C, fibre and flavonoid.

MATERIALS AND METHODS

C. suhuiensis callus initiation

The preparation of *C. suhuiensis* cotyledon as the explant for initiation of callus culture and its media preparation was carried out according to Fathil *et al.* (2017). The excised cotyledons were cultured on solid medium to initiate callus and kept in a plant growth chamber (Lab-Line Instruments, Inc. USA) at 25 ± 2°C under the dark condition. The callus cultures were maintained by subculturing them for every 4 weeks on solid media (Murashige and Skoog (MS) (Duchefa, USA) medium supplemented with 500 mg/L malt extract (Friendemann Schmidt, Germany), 30 g/L sucrose (R&M Chemicals, UK), 0.5 mg/L 2,4-D (R&M Chemicals, UK) and gelrite (Duchefa, Netherlands)) until the callus became friable and used for establishing cell suspension cultures.

Establishment of *C. suhuiensis* cell suspension culture

Friable callus (3 g) of *C. suhuiensis* were transferred to a flask containing 100 mL of fresh media. The media composition was the same as the callus maintenance media but without gelrite. The cultures were incubated at 100 rpm on an orbital shaker (SK-71 Lab Companion, South Korea) in a culture room with a regulated temperature of 25 ± 2°C and maintained under 16/8 photoperiod light condition provided with the cool white fluorescent lamps (Philips, 2500 lm). For every three weeks, the cells were subcultured by pipetting 30 mL of spent media containing cells into 250 mL Erlenmeyer flask with 70 mL of fresh media.

Manipulation of culture medium components

MS medium supplemented with 30 g/L sucrose, and 500 mg/L malt extract was prepared as the basic medium. Different PGRs and carbon sources were added before autoclave depending on the set objective.

The first aim was to determine the best type of carbon source for *C. suhuiensis* suspension growth. Four different carbon sources (sucrose, glucose, fructose and glycerol) were tested at the concentrations of 30 and 50 (g/L), respectively. The type of PGR which is 0.5 mg/L 2,4-D and 10% (v/v) (approximately 1 g DW/L) of the cells were added as the constant parameters when preparing the medium before autoclave (Fathil *et al.*, 2017).

Secondly, for the PGRs effect, 0.5, 1.0, 2.0 (mg/L) 2,4-D and its combination with benzylaminopurine (BAP) (R&M Chemicals, UK) and kinetin (R&M Chemicals, UK) at 0.5 mg/L and 1.0 mg/L, respectively were selected. Sucrose was set as constant at 30 g/L as well as 10% (v/v) of inoculum size.

Three inoculum sizes were studied which were 10, 20 and 30 (% (v/v)). The cells that are at their exponential phase of growth (18th day of incubation) were used as the inoculum (Puad *et al.*, 2018). In order to make a final volume of 20 mL, *C. suhuiensis*

cells at 2, 4 and 6 (mL) were added into the new fresh media prepared at 19, 18 and 17 (mL), respectively. Sucrose at 30 g/L and 0.5 mg/L 2, 4-D were added as constant parameters (Singh *et al.*, 2017).

After adjusting the pH to 5.7 ± 0.1, the media used for all experiments were autoclaved at 20 psi and 121°C for 15 minutes for sterilization. All cultures were placed on an orbital shaker (SK-71 Lab Companion, South Korea) with a speed of 100 rpm in a culture room with regulated temperature of 25 ± 2°C m under 16/8 photoperiod light condition provided with the cool white fluorescent lamps (Philips, 2500 lm).

Cell growth analysis

Sampling was done for every three days in 24 days of cultivation period by taking three flasks (to represent triplicates) containing *C. suhuiensis* suspended cells. The cells in each flask were harvested by filtering the cultures through a pre-weighed filter paper (Filtres Fioroni 601, ∅ 90 mm) as the initial weight using a filter funnel (90 mm) connected to a vacuum pump (Fisher, FB 70155). Then, the cells were placed in an oven (Memmert, Germany) for 24 hours at 60°C for the cell dry weight (CDW) measurement. After few hours, they were weighed again as the final weight to ensure that the weight is constant and there was no more water within the cells. CDW was calculated by subtracting the final weight to initial weight of the filter paper.

RESULTS AND DISCUSSION

Effect of carbon sources on *C. suhuiensis* suspension growth

C. suhuiensis suspension cultures were successfully established and achieved significant CDW and μ_{max} values especially for sucrose and glucose as the sole carbon source, respectively. The highest CDW and μ_{max} were recorded for 50 g/L glucose-grown cells. The maximum specific growth rate, μ_{max} was obtained from the plot of the natural logarithm (ln) CDW versus time from the CDW data in the log phase. Based on the calculated μ_{max} as shown in Table 1, cells were grown at a higher rate when sucrose and glucose were supplied as the carbon sources, respectively while cells grown on fructose and glycerol exhibited a slower growth rate. Table 1 also shows that *C. suhuiensis* cells achieved the highest CDW and μ_{max} which was 23.2 g/L and 4.47 h^{-1}, respectively on 50 g/L glucose. The CDW was 22.68 times higher than initial weight. Although the μ_{max} values for 30 g/L and 50 g/L glucose were similar, the CDW achieved was lower for 30 g/L glucose.

In this study, sucrose at 50 g/L (17.9 g/L, eight times higher than initial weight) gave a lower CDW than glucose at the same concentration. Although sucrose has been the major carbon source used in the cell cultures, in some cases, it may cause hypoxia and ethanol accumulation in the cells due to its quick metabolization (Ramarosandratana *et al.*, 2001; Jalil *et al.*, 2015). Thus sucrose is totally or partially replaced by other types of carbon sources (Jalil *et al.*, 2015). The lowest CDW and μ_{max} were recorded for 50 g/L fructose and 30 g/L glycerol, respectively. Various effects of carbon sources on *C. suhuiensis* cell growth can be related to the sensitivity of this species to tested carbon sources or the product generated during its metabolism. Besides, it can be deduced that the existing metabolic pathways for utilization of fructose and glycerol as a sole carbon source, respectively might not able to maintain adequate production levels of intracellular precursor carbon chain and energy since an adequate level of gluconeogenic enzymes is needed (Mello *et al.*, 2001).

There are limited reports on the effects of different carbon sources on the cell suspension cultures of Citrus species including *C. suhuiensis*. A study carried out by Agisimanto *et al.* (2011) investigated the effects of sucrose, glycerol and a combination of sorbitol and galactose on *C. suhuiensis* cell suspension cultures for somatic embryogenesis. It was observed that cells grown on 50 g/L sucrose as the carbon source achieved the

Table 1. Maximum CDW and μ_{max} on *C. suhuiensis* cell suspension cultures on different types and concentrations of carbon sources. Data are presented as mean ± SD (n=3 replicates)

Carbon Sources	Concentration (g/L)	μ_{max} (h^{-1})	Maximum CDW (g/L)
Sucrose	30	0.316	11.90 ± 0.031
	50	0.366	17.90 ± 0.041
Glucose	30	0.467	10.70 ± 0.0145
	50	0.462	20.60 ± 0.090
Fructose	30	0.219	2.72 ± 0.014
	50	0.171	2.12 ± 0.004
Glycerol	30	0.155	3.48 ± 0.006
	30	0.213	4.20 ± 0.007

highest cell density compared to other carbon sources. In addition, glycerol was observed to be ineffective in increasing *C. suhuiensis* cell density. However, BAP was used as the PGR while in this study, 2,4-D was used due to its usage in the majority of the previous studies on other Citrus species (Azim *et al.*, 2013; Gerolino *et al.*, 2015; Kazmi & Mirbahar, 2015).

For other species of dicotyledon plants, Heidarifar and Nayeri (2015) examined the effects of sucrose and glucose at the concentration of 20, 30 and 50 g/L, respectively on *Sesamum indicum* L. species, the famous oilseed crops. It was concluded that the medium supplemented with 30 g/L sucrose under the dark condition and agitation speed of 130 rpm was more efficient compared to the ones grown under the light condition. However, in this study the effects of light irradiation and agitation speed were not investigated. A study on *Pueraria tuberosa* (*P. tuberosa*), a perennial woody plant (Karwasara & Dixit, 2013) reported sucrose as the best carbon source for the highest biomass followed by maltose. Also, glucose and maltose gave a high biomass density up to day ten but sucrose led to achieve a higher biomass after day 15 while fructose and galactose were less suitable for the growth of *P. tuberosa* suspension cultures.

Thanh *et al.* (2007) studied on different concentrations of sucrose (0, 20, 30, 40, 50, 60, and 70 g/L) on *Panax vietnamensis (P. vietnamensis)* suspension cultures and the optimal cell growth was achieved in the range of 30 to 50 g/L sucrose. A higher concentration of sucrose inhibited *P. vietnamensis* suspended cell growth. Cui *et al.* (2010) stated that 20–30 g/L sucrose have frequently reported as the optimal concentration for suspension culture and higher concentration will repress the growth. Besides, the reduction in a cell or root growth might be related to the sucrose-induced osmotic stress when the cultures are supplied with the high levels of sucrose (Cui *et al.*, 2013) and also might cause cell dehydration and therefore reduced cells proliferation (Jalil *et al.*, 2015).

Effect of PGRs on *C. suhuiensis* suspension growth

Under *in vitro* condition, PGR is also one of the fundamental elements in manipulating the organogenic response in any plant tissue cultures (Jayaraman *et al.*, 2014). Based on the results, cells in set A media (Table 2) recorded the highest CDW and μ_{max} which were 11.91 g/L (7.22 times higher than initial weight) and 0.3161 h^{-1}, respectively. A higher concentration of 2,4-D gave a negative effect on the cell growth in which only 2.91 g/L and 3.06 g/L dry weight were obtained for set B and C, respectively. Lower CDW was also observed when 2,4-D was combined with BAP and kinetin as depicted in Table 2. Besides, the second highest CDW and μ_{max} for the combination of 2,4-D with either BAP and kinetin was achieved by set G where 4.57 g/L CDW and 0.227 h^{-1} for μ_{max} were measured.

A single PGR may regulate a wide range of physiological and growth processes and on the other hand, the action of many PGRs may regulate particular process, thus the response may differ in each species of plant (Santoro *et al.*, 2013). In this study, the effect of auxin alone greatly influences the growth on *C. suhuiensis* suspension cultures where the highest CDW (11.91 ± 0.031 g/L) was achieved in 0.5 mg/L 2,4-D medium. The result obtained is contradicted with a study by Agisimanto *et al.* (2011) which reported that the highest growth rate of *C. suhuiensis* suspension cultures (6.69 mg/day) was on 1.5 mg/L BAP whereas in this study medium with BAP inhibited the growth of *C. suhuiensis* cultures. Nevertheless, their study was focusing on somatic embryogenesis production where BAP is one of the important factors for the regeneration of Citrus somatic embryos (Gholami *et al.*, 2013).

In this study, there was no investigation on secondary metabolite production and 2,4-D alone is sufficient to promote high cell growth of *C. suhuiensis* suspension culture. This is supported by a previous study by Lian *et al.* (2002) on the effect of PGRs on cell growth and saponin production of *Panax Ginseng*. The study revealed that maximum biomass yield was obtained in the

Table 2. Maximum CDW and μ_{max} on *C. suhuiensis* cell suspension cultures in different types and concentrations of PGRs. Data are presented as mean ± SD (n=3 replicates)

Set	Concentration of PGRs (mg/L)			Maximum CDW (g/L)	μ_{max} (h^{-1})
	2,4-D	Kinetin	BAP		
A	0.5	–	–	11.91 ± 0.031	0.32
B	1.0	–	–	2.91 ± 0.005	0.13
C	2.0	–	–	3.06 ± 0.007	0.13
D	2.0	1.0	–	3.86 ± 0.003	0.19
E	1.0	0.5	–	2.96 ± 0.012	0.12
F	2.0	–	1.0	2.60 ± 0.006	0.13
G	1.0	–	0.5	4.57 ± 0.025	0.23

medium containing 1 mg/L 2,4-D while secondary production (saponin) was higher in the medium comprising of 7 mg/L IBA with addition of cytokinin (0.5 mg/L BA or kinetin).

During cellular growth, auxin can stimulate the acidification of cell wall that results in increasing extensibility and also inducing the transcription of specific mRNAs that code for proteins associated with cellular growth (Majda & Robert, 2018). Interaction between auxin and cytokinin can control many aspects in cell growth, cell differentiation, and organogenesis in tissue and organ cultures. The suitable concentration of each PGR varies greatly according to the type of plant cultured, cultural conditions, and the type of PGR used. Both auxin and cytokinin are usually essential for growth and morphogenesis. However, auxin can inhibit cytokinin accumulation, whereas cytokinins can inhibit some of the actions of auxin (Gaspar *et al.*, 1996; Kotov & Kotova, 2018).

Effect of inoculum size on *C. suhuiensis* suspension growth

The CDW and μ_{max} were at the highest when 10% (v/v) of inoculum was cultured into a fresh medium where 15.45 ± 0.005g/L and 0.711h^{-1}, respectively were recorded as shown in Table 3. Lower CDW and μ_{max} were observed on 20% (v/v) and 30% (v/v) of inoculum sizes. It shows that low inoculum size (10% (v/v)) is preferable for *C. suhuiensis* cell growth. Besides, the cells in 10% (v/v) of inoculum achieved 18-folds increment than the initial concentration (0.828 g/L). Meanwhile, only 6-folds and 5-folds cell increment were observed in 20% (v/v) and 30% (v/v) of inoculum size, respectively. This result showed that a lower inoculum size gave a maximum cell weight and this is aligned with a finding reported by Lo *et al.* (2012) where the highest growth index (38.3) of *Artemisia annua* suspension culture was obtained when using the lowest inoculum size (0.625% (w/v)). At a higher density, the growth of cells would decrease due to the increasing limitation of key nutrients including oxygen (Hwang *et al.*, 2004).

A study on *Stevia rebaudiana* suspension cultures by Mathur and Shekhawat (2013) investigated three different inoculum sizes; 5, 10 and 20 (g/L) fresh weight. The optimal growth response was obtained when 10 g/L of inoculum was cultured by a significant increase in packed cell volume (PCV) after day 14 and the cell growth was unfavourable at lower (5 g/L) and higher (20 g/L) inoculum density. The study also concluded that the optimal inoculum density has significant effects on the productivity and growth kinetics because of the stimulatory influence of inoculum density in plant tissue cultures.

Table 3. Maximum CDW and μ_{max} on *C. suhuiensis* cell suspension cultures using different inoculum sizes. Data are presented as mean ± SD (n=3 replicates)

Inoculum size (v/v) (%)	Maximum CDW (g/L)	μ_{max} (h^{-1})
10%	15.45 ± 0.005	0.711
20%	11.24 ± 0.007	0.306
30%	13.16 ± 0.013	0.436

Inoculum sizes ranged from 2.5% (w/v) until 10% (w/v) were tested on cell suspension culture of *Corydalis saxicola* Bunting, a rare medicinal plant to determine their effect on biomass and alkaloid production. The highest value of biomass was measured at 13.1 g/L on day 21 when using 10% (w/v) of inoculum which increased the biomass 6 times compared to initial one (2.1 g/L). A lower inoculum size demonstrated a longer lag phase and slower growth (Cheng *et al.*, 2006). During the lag phase, the adaptation is required for the cells to exploit in the new environmental condition, to repair macromolecular damages and to the synthesis of cellular components needed for the growth of cell (Rolfe *et al.*, 2012).

In contrast to this present study, Yang *et al.* (2009) showed that a low inoculum density of 5 g fresh weight/L gave a low biomass and flavonoid production by the fresh *Glycyrrhiza inflata* cells. Meanwhile the highest inoculum density (50 g fresh weight/L) reached a maximum of 18.2 g DW biomass and 58.7 mg/L of flavonoid production. Inoculum density is an important factor which can influence not only the cell growth, but also the accumulation of secondary metabolite. The disparity in cell inoculum density could cause big changes in culture parameters such as concentrations of dissolved oxygen and dissolved gaseous metabolites with the related enzyme activities and these changes could further affect the cell metabolism directly or indirectly (Yang *et al.*, 2009).

CONCLUSION

Cell suspension culture of *C. suhuiensis* was successfully established by manipulating different types and concentration of carbon sources, PGRs and inoculum size. The highest cell growth was achieved when glucose at 50 g/L was supplemented to the media with 0.5 mg/L 2,4-D at 10% (v/v) of inoculum. Other effects such as agitation speed, pH value, and presence of light can be further explored for the enhancement of cell dry weight as well as for the secondary metabolite production in *C. suhuiensis* cultures.

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