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Optimization of plant growth regulators for *Citrus suhuiensis* callus induction

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Abstract. Correct types and concentration of plant growth regulators (PGRs) will enhance and optimize the growth of callus cultures. This paper reported the effects of several types of cytokinins (2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthalene acetic acid (NAA)) and auxins (6–Benzylaminopurine (BAP) and kinetin) on the callus induction of *C. suhuiensis.* The cotyledons from *C. suhuiensis* seeds were excised as the explant and cultured on Murashige and Skoog (MS) media, 3% (w/v) sugar, 0.05% (w/v) malt extract and 0.25% (w/v) agar under the continuous dark condition supplemented with the chosen PGRs at the concentration range of 0.5, 1.0, 2.0, 3.0 and 4.0 mg/L. The growth of callus at each treatment was measured as gram (g) of fresh weight and percentage of callus induction. The results showed that 1.0 mg/L 2,4-D gave the highest growth of callus (0.15 g and 100% callus percentage). After identifying the effective PGRs, Central Composite Design (CCD) from the Design Expert® software version 9.0 was used to obtain the optimum concentration of cytokinin and auxin on *C. suhuiensis* callus cultures. It was observed that the highest amount of callus culture induced was 0.218 g when the media was supplemented with 1.0 mg/L 2,4-D and 1.0 mg/L kinetin.

Keywords: : Central composite design (CCD), *Citrus suhuiensis*, callus culture induction, plant growth regulators (PGRs)

INTRODUCTION

Rutacea as the genus family of citrus fruits is an edible fruit that are known for their high content of vitamin C and become the most reliable natural source of vitamin C (Ezeigbo *et al.*, 2013). They are also a good source of carbohydrates, dietary fiber and minerals (Kazmi *et al.*, 2015) and widely consumed to prevent flu, colds and to support the immune system (Kasprzyk-pawelec, Pietrusiewicz, and Szczuka 2015). Throughout the world, citrus ranked the first place in the production of fruits crops and they have been extensively grown in many countries regardless of the climate condition such as Brazil, USA, China and Mexico (Ladanyia, 2010). In Malaysia, *C. suhuiensis* or commonly known as limau madu is cultivated for local consumption and have been planted in the states of Terengganu, Kedah, Pahang, Perak, Johor and Sarawak (Elcy *et al.*, 2012). However, *C. suhuiensis* is known to be liable to diseases caused by Fusarium, Phytophthora and Colletotrichum as well as insects and pests that decrease the fruit production or trees (Noor *et al.*, 2009). Currently, one of the potential alternatives in biotechnology field is plant cell culture technique or *in vitro* micropropagation where the cell cultures can be established regularly under sterile conditions from explants, such as plant leaves, seeds, hypocotyl, stems, roots, and meristems for organogenesis, multiplication and also callogenesis. This technique has proven to give great impacts on the agriculture and industrial that provide the required amount of plants needed to meet the increasing world demand which allows the production and propagation of genetically homogeneous, disease-free plant material in a shorter time compared to conventional breeding. Besides, micropropagation technology has a huge potential to produce the high quality of plant, disease resistance and stress tolerance capabilities (Hussain *et al.* 2012). Callus culture can be established from a single differentiated cell and these cells are totipotent in which they are able to regenerate the whole plant body. The intermediate ratio of auxin and cytokinin group will promote callus induction while a high ratio of auxin -to-cytokinin or cytokinin-to-auxin initiate root and shoot regeneration, respectively (Ikeuchi *et al.*, 2013). The production of callus cultures and the regeneration of plant are the main steps in *in vitro* biotechnology manipulation (Savita *et al.*, 2011). 2,4-D is known to be effectively used as plant growth regulator for callus formation and its optimum concentration depends on the types of plant species (Abdullah *et al.,* 2013). However,

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very few literatures can be found on cell cultures of Citrus sp., especially on *C. suhuiensis.* To the best of our knowledge, this is the first study done on the effects and optimization of PGRs on callus cultures for this species. Besides, reports on the application of Design of Experiment (DOE) in plant cell culture are limited and only several studies were found on Citrus sp. where the media composition (Niedz and Evens 2008) and culture condition factors on the cultivation of plant cell culture were investigated (Yaacob *et al*. 2014). Therefore, this study was carried out to determine the best type and concentration of PGR that can induce the callus cultures of *C. suhuiensis* and optimization of the callus induction using CCD.

MATERIALS AND METHODS

Plant Material. *C. suhuiensis* fruit was bought at a local shop in Gombak, Selangor and the seeds of the fruits were collected. Some of the seeds were cultivated on the soil to obtain small *C. suhuiensis* plant and then sent to the Institute of Bioscience, University Putra Malaysia (UPM) for species identification and confirmation.

Preparation of explant. *C. suhuiensis* seeds were surface sterilized by firstly immersing them in 70% (v/v) ethanol for 5 minutes and 20% (v/v) of NaOCl for 20 minutes to remove any disinfectants. Next, the seeds were washed with sterile distilled water for 3 times to ensure that no chemical residues left. The seed's coat was removed by using scalpel and forceps and the cotyledon was collected as the explant. The cotyledons were cut into several slices and each slice was weighed and taken as the initial weight. After 4 weeks, the explants were weighed again and the reading was recorded as the final weight. The weight of the callus was determined by subtracting the final with the initial weight. All steps were done under the laminar flow cabinet.

Media Preparation. Murashige and Skoog (MS) (Duchefa, USA) media supplemented with 0.05% (w/v) malt extract (Friendemann Schmidt, Germany) and 3% (w/v) sucrose (R&M, India) was used. After adjusting the pH to 5.7 ± 0.1, 0.25% (w/v) gelrite (Duchefa, USA) was added to the mixture for solid medium preparation. The medium was autoclaved at 20 psi and 121°C for 15 minutes for sterilization. The medium was left to cool down before pouring 25 ml of it into each 90mm x 15mm petri dish which was then solidified and sealed with parafilm (Azim *et al.*, 2013).

Effect of PGRs on callus induction. Two groups of PGRs which are auxins and cytokinins were tested for *C. suhuiensis* callus induction. 2,4-D (PhytoTechnology Laboratories, USA) and NAA (PhytoTechnology Laboratories, USA) from auxin group and BAP (PhytoTechnology Laboratories, USA) and kinetin (PhytoTechnology Laboratories, USA) from cytokinins group were selected. The concentrations of PGRs used were 0.5, 1.0, 2.0, 3.0 and 4.0 mg/L, respectively and 0.0 mg/L as the control. Four explants were placed on each petri dish and 8 replicates were done for each treatment. All petri dishes were incubated in plant growth chamber at 25±2°C under the continuous dark condition. The callus percentage was expressed as a percentage of total number explant that produce callus per total number of explant cultured while the fresh weight of the callus culture were harvested and measured after 4 weeks (Sahraroo, Babalar, and Hossein 2014).

Optimization of callus induction using Central Composite Design (CCD) A CCD of RSM from Design Expert® software 9.0 (Stat-Ease, Minneapolis, MN) was developed by choosing the concentration of cytokinin that gave the best callus induction to optimize the concentration of the combination of auxins and cytokinins. Two sets of CCD were carried out to determine which cytokinins (BAP and kinetin) were more favored with auxin (2,4-D) for callus cultures optimization. The experimental range for both variables (A: auxin and B: cytokinin) was 0.5 mg/L for minimum value and 1.5 mg/L for maximum value for two factors and 3 level design. The design was set up with three repetitions at the central point and face centered alpha. The replicates at central point are relevant to measure the error of the model and to estimate the curvature of the response function (Gomez-Montes *et al*. 2015). Thus, 11 runs were set up by CCD. The explants were incubated under the same conditions as in the effect of PGRs on callus induction. After 4 weeks, the fresh weight of callus cultures was recorded. The data were processed by using quadratic model including ANOVA analysis to evaluate the significance of the factors and their interactions (Abbasi, Hooshyar, and Baghereih-najjar 2016).

RESULTS AND DISCUSSION

The effects of PGRs on callus induction The capability of callus cultures formation depends on the type of PGRs, their concentrations and combinations in the growth medium (Sheeba *et al.*, 2013). Based on the results shown in Table 1, 2,4-D achieved the highest callus growth (0.15 g) at 1.0 mg/L with 100 % of callus percentage. Meanwhile for NAA, the highest callus growth (0.12 g) was at the concentration of 2.0 mg/L but it is still lower than the callus supplied with 2,4-D. This result was slightly different from Azim *et al.* (2013) in which they reported that the highest growth of Malta (*C. sinensis)* callus was obtained when using 2.0 mg/L 2,4-D. Another study by Savita *et al.*, (2015) also gave the same result for *C. jambhiri* where the maximum callus response (in percentage) was obtained when 2.0 mg/L 2,4-D was

supplemented in MS medium.

In terms of callus morphology, it was observed that *C. suhuiensis* callus produced from 2,4-D was yellowish and soft while in NAA, the callus was hard and compact. Besides that, using NAA as PGR for *C. suhuiensis* has resulted in the root production for the concentration of 1.0 until 4.0 mg/L. Figure 1 illustrated the response of the explants towards each PGR. A study carried out on the same species by Noor *et al*. (2009) showed that NAA alone gave root formation and BAP gave shoot formation. In this study, there were no responses observed for BAP and kinetin since the culture was placed completely in the dark condition and this is in agreement with the earlier report that only shoots were produced when BAP and kinetin were supplied as PGRs on *C. limon* under the photoperiod condition (Goswami *et al*., 2013).

Optimization of callus induction After experiments on callus induction with different PGRs were carried out, the highest growth using 1.0 mg/L 2,4-D was combined with cytokinins (BAP and kinetin) for its optimization using CCD. From the results, the combination of 2,4-D and kinetin showed a better response compared to 2,4-D with BAP. The growth of callus in 1.5 mg/L 2,4-D with 1.5 mg/L

Table 1. Effect of PGRs on callus induction of *C. suhuiensis*

Hormone	Concentration (mg/L)	Responses	Callus percentage (%)	Callus weight (g)
Control	-	No response	-	-
2,4-D	0.5	Callus	100	0.107
	1.0	Callus	100	0.154
	2.0	Callus	100	0.100
	3.0	Callus	100	0.116
	4.0	Callus	100	0.123
NAA	0.5	Callus	75.0	0.072
	1.0	Callus & root	87.5	0.099
	2.0	Callus & root	100	0.118
	3.0	Callus & root	100	0.110
	4.0	Callus & root	100	0.970
BAP	All	No response	-	-
Kinetin	All	No response	-	-

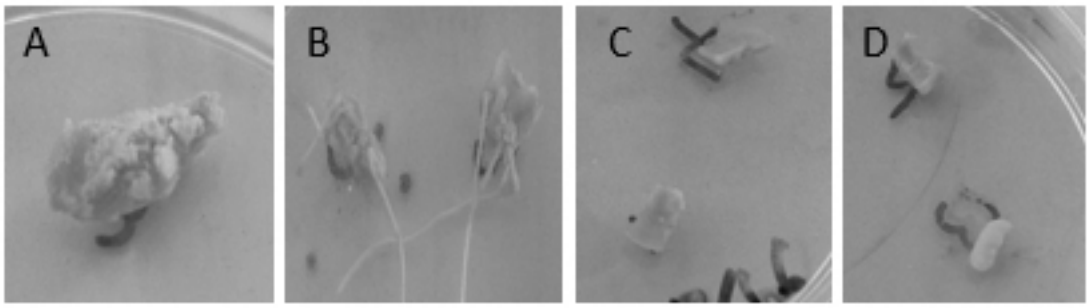

Figure 1. Callus response on each PGRs. **A:** callus culture in 2,4-D, **B:** callus and root culture in NAA, **C:** No response in BAP, D: No response in kinetin

Table 2. Data from CCD for combination of 2,4-D with BAP

Run	Factor A: 2,4-D mg/L	Factor B: BAP mg/L	Response 1: Fresh weight g
1	1.00	0.50	0.13
2	1.00	1.00	0.133
3	1.00	1.00	0.13
4	1.50	1.50	0.184
5	0.50	1.00	0.169
6	1.50	0.50	0.139
7	1.00	1.50	0.153
8	1.00	1.00	0.13
9	0.50	0.50	0.173
10	1.50	1.00	0.147
11	0.50	1.50	0.169

BAP gave the highest weight (0.184 g) as recorded in Table 2. Based on ANOVA analysis (Table 3), the generated model was significant since the p value < 0.05 and the lack of fit was not significant (p value > 0.1). ANOVA analysis has the ability to describe the relationship between the variables (PGRs) on the response of callus induction (Bagherieh-Najjar & Nezamdoost, 2016). Consequently, the model showed that it agreed with the experimental results as the response to the variable. The standard deviation of this model was 0.00356 and the R^2 was 0.9843 which was close to 1 indicate that the model fitted well with the experimental data (Gomez-Montes *et al*. 2015). However the three-dimensional (3D) response surface plot in Figure 2 was not optimized since the curve plot was not at the centre. This suggests that the chosen concentration ranges for 2,4-D and BAP need to be either more than 1.5 mg/L or less than 0.5 mg/L since the second highest weight was at 0.5 mg/L 2,4-D and 0.5 mg/L BAP.

For the combination of 2,4-D and kinetin, the optimum value was at 1.0 mg/L 2,4-D and 1.0 mg/L kinetin where the fresh weight recorded was 0.218 g and this value was higher than using 2,4-D alone as stated in Table 4. As shown in Figure 3, the 3D plot illustrates the optimum concentration of 2,4-D and kinetin. From the ANOVA

Table 3. ANOVA analysis for optimization of callus induction for 2,4-D and BAP from CCD

Source	Squares	df	Square	Value	Prob > F	
Model	3.967E-003	5	7.934E-004	62.61	0.0002	significant
A-2,4-D	2.802E-004	1	2.802E-004	22.11	0.0053	
B-BAP	6.827E-004	1	6.827E-004	53.87	0.0007	
AB	6.002E-004	1	6.002E-004	47.37	0.0010	
A^2	1.688E-003	1	1.688E-003	133.23	< 0.0001	
B^2	2.199E-004	1	2.199E-004	17.35	0.0088	
Residual	6.336E-005	5	1.267E-005			
Lack of Fit	5.736E-005	3	1.912E-005	6.37	0.1386	not significant
Pure Error	6.000E-006	2	3.000E-006			
Cor Total	4.031E-003	10				

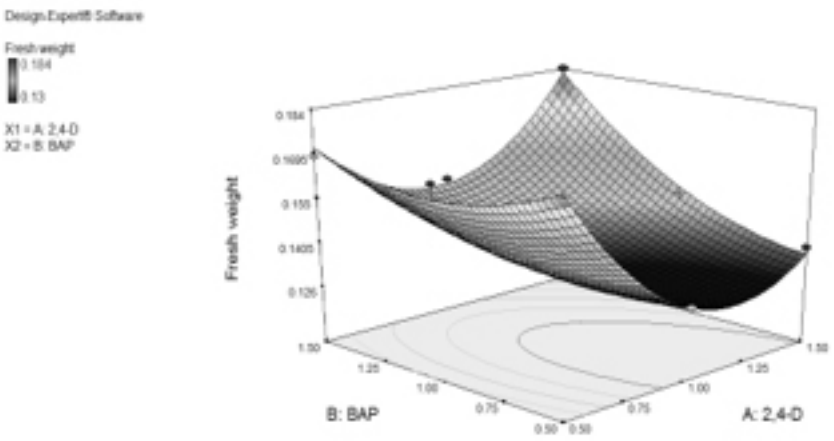

Figure 2. The 3D surface from CCD for the combination of 2,4-D and BAP

Table 4. Data from CCD for combination of 2,4-D with kinetin

Run	Factor A: 2,4-D mg/L	Factor B: BAP mg/L	Response 1: Fresh weight g
1	1.00	1.50	0.155
2	1.00	1.00	0.207
3	1.00	0.50	0.174
4	0.50	1.00	0.17
5	1.50	1.00	0.16
6	0.50	0.50	0.165
7	1.50	1.50	0.132
8	1.50	0.50	0.161
9	1.00	1.00	0.213
10	1.00	1.00	0.218
11	0.50	1.50	0.148

analysis (Table 5) that used Response Surface Quadratic Model, it is proven that the model was significant (p value < 0.05) and the lack of fit was not significant (p value > 0.1). Hence, it can be concluded that the model was sufficiently in agreement with the experimental data. Besides that, standard deviation was 0.013 and the R^2 was 0.8942 with R2adj equal to 0.7884. When the R^2_{adj} is close to R2, it suggested that the model is also significant (Abbasi, Hooshyar, and Bagherieh-najjar 2016)

Ramdan *et al.* (2015) reported that the combination of 2,4-D and BAP at 1.0/0.5 and 2.0/1.0 (mg/L), respectively achieved the highest amount of callus induction from five different types of citrus rootstock. Kazmi *et al.* (2015) also concluded that 2.5 mg/L 2,4-D with 0.5 mg/L BAP gave the highest growth of callus for Kinnow Mandarin species. However, these studies did not cover the combination effect of 2,4-D and kinetin. There was a study on callus induction of *C. jambhiri* for the combination of 2,4-D with BAP as well as 2,4-D and kinetin. Based on the results, the combination of 2,4-D and BAP gave a higher response (83.33%) at the concentration of 2.0 mg/L and 0.75mg/L, respectively while 2.0 mg/L 2,4-D with 0.75 mg/L kinetin gave a lower response (58.33%) (Savita *et al.*, 2011). A recent study by Kasprzyk-pawelec *et al.* (2015) on *C. limon* species concluded that the highest percentage of callus induction (97%) was obtained when using MS supplemented with 1.0 mg/L 2,4-D plus 0.2 mg/L NAA while the combination of 2,4-D and kinetin gave a slightly lower percentage which was 70%.

Table 5. ANOVA analysis for optimization of callus induction for 2,4-D and kinetin from CCD

Source	Sum of Squares	df	Mean Square	F Value	P value Prob > F	
Model	6.973E-003	5	1.395E-003	8.45	0.0176	significant
A-2,4-D	1.500E-004	1	1.500E-004	0.91	0.3841	
B-Kinetin	7.042E-004	1	7.042E-004	4.27	0.0937	
AB	3.600E-005	1	3.600E-005	0.22	0.6601	
A^2	2.193E-003	1	2.193E-003	13.29	0.0148	
B^2	2.268E-003	1	2.268E-003	13.75	0.0139	
Residual	8.250E-004	5	1.650E-004			
Lack of Fit	7.644E-004	3	2.548E-004	8.40	0.1082	not significant
Pure Error	6.067E-005	2	3.033E-005			
Cor Total	6.973E-003	10				

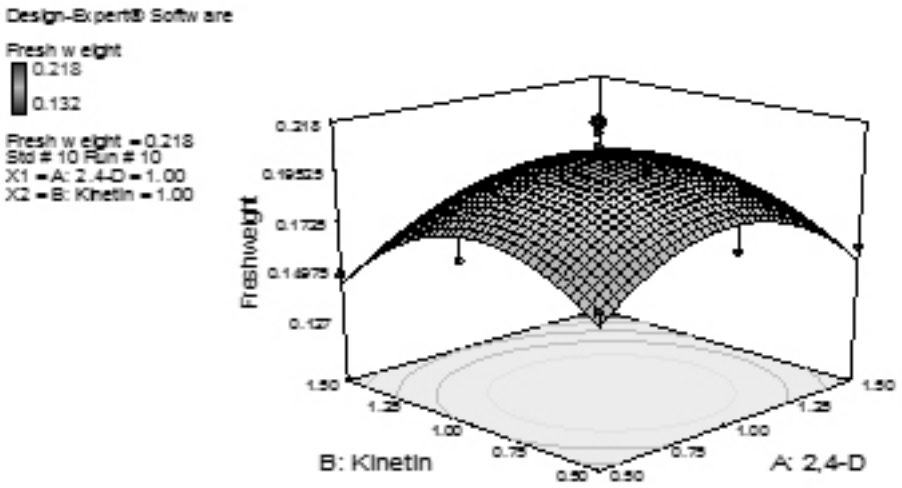

Figure 3. The 3D surface from CCD for the combination of 2,4-D and kinetin

CONCLUSION

In conclusion, *C. suhuiensis* callus culture has been successfully induced from its cotyledon. For *C. suhuiensis* callus induction, 2,4-D gave the best response in which the highest weight recorded for 1.0 mg/L of 2,4-D alone was 0.15 g. Furthermore, its combination with kinetin and BAP has promoted better callus culture production by using a statistical modeling approach (CCD-RSM). The optimization of 2,4-D with kinetin gave the optimum value of callus weight which was 0.218 g and it is recommended to use a wider range of BAP concentrations to optimize the amount of callus for the combination of 2,4-D and BAP.

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