OPTIMIZATION OF SURFACE STERILIZATION METHOD AND INITIATION OF BITTER CASSAVA CALLUS CULTURE

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ABSTRACT: Surface sterilization is essential in explant preparation for *in vitro* cultivation to reduce contamination risk. This study focused on optimizing surface sterilizing technique to initiate bitter cassava callus culture (Rayong cultivar) for starch production based on the effects of plant growth regulator (PGR). Bitter cassava is widely used in the food and non-food industries thus, its demand is forecasted to be high in the next several years. Farmers faced many challenges in large-scale cassava plantations such as lack of infrastructure, cassava diseases and poor climatic conditions. Plant tissue culture is proposed due to its advantages such as high yield in a shorter time compared to the traditional method and the cultivation can be done outside the plant's season. Soaking time in 70% ethanol (min), concentration of sodium hypochlorite (%) and soaking time in sodium hypochlorite (min) were selected for optimization of surface sterilizing condition of cassava explants (leaf and stem). Face Centered Central Composite Design (FCCCD) under Response Surface Methodology (RSM) in Design-Expert v9.0.6. was used for designing experiments and optimization purpose to minimize the percentage of contamination. Next, different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) (5, 10, 15 and 20 mg/L) were supplemented to Murashige and Skoog (MS) medium to investigate their effect on callus initiation. Based on the results, soaking cassava explants for 5 minutes in 70% ethanol followed by 10 minutes in 5.75% in sodium hypochlorite gave the least percentage of contamination (16.67% for the leaf and 25% for the stem). The highest frequency of callus formation (41.67%) was achieved when using 5 mg/L of 2,4-D from stem explant. The results from this study can serve as a starting point in establishing bitter cassava callus culture for starch production.

KEY WORDS: *Manihot esculenta Crantz, Rayong, surface sterilization, callus culture, 2,4–dichlorophenoxyacetic acid.*

1. INTRODUCTION

Cassava or manioc (*Manihot esculenta* Crantz) is currently one of the biggest food crops in Africa, Asia and Latin America [1]. Cassava is the 5th most important staple crop globally after maize, rice, wheat, and potato concerning production and caloric intake [2]. There are two types of cassava which are sweet and bitter cassava. The main difference between these two types is the content of the antinutritional values such as cyanide. Bitter cassava contains more antinutritional values compared to the sweet cassava. When the

cassava is processed, the toxic cyanogenic glucosides can be removed or reduced, usually by boiling process and the palatability can also be improved [3].

Concerning cassava's important roles in various industries, large-scale production of cassava has gained the interest of many farmers. Unfortunately, this incentive is not practical as it needs several thousand hectares of land. These lands are usually located in the rural areas which cause several obstacles such as transportation cost to transfer them to the processing factories which are usually located in the urban areas [4]. Moreover, cassava plant is exposed to cassava diseases such as the leaf spot and cassava bacterial blight disease (CBB) that halt its growth and production. Usually, it takes around 12 months before the cassava can be harvested for their starch which is considered as a long period.

Therefore, plant culture technology is proposed as a potential solution for these challenges. It has many advantages such as the ability to shorten the time for plant growth and production and the space needed for the cultures is smaller compared to the plantations. As an example, this technology has enabled the farmers to produce large quantities of clean banana planting materials that can mature in 12-16 months compared to 2-3 years by the conventional banana [5]. An initiation of cassava callus culture is suggested because callus culture is the starter of any plant tissue culture. Callus is a non-specialized, non-organized and dividing mass of cells. Most of the part of a plant can be used as an explant to initiate callus culture. There are many factors involved in plant cell growth and development [6]. For the initiation of callus culture, surface sterilization techniques, as well as types and concentrations of plant growth regulators (PGRs) play important roles.

According to Putri et al. (2019) [7], the constant problem for plant tissue culture is the microbial contamination in which a higher chance of contamination occurs when the explants are taken directly from the field. The contaminants can be eliminated or killed by surface sterilization process or technique which does not give any effect on the biological activity of the explants and it is the most crucial step in the plant tissue culture [8].

Meanwhile, auxins and cytokinins or a combination of both in balanced amounts are typical PGRs used to induce callus culture. Sometimes, gibberellin (GA) is also added depending on the types of plant used. Based on the literature, the most frequently used auxin to initiate callus culture is 2,4-dichlorophenoxyacetic acid (2,4-D) [6]. Thus, in this study, the optimization of surface sterilization techniques on bitter cassava explants (leaf and stem) was conducted to achieve a low percentage of contamination by varying the concentration of sodium hypochlorite (1.5-10%) and the soaking time (5-10 min) as well as the soaking time in 70% ethanol (1-5 min) and to establish the callus culture from the selected cassava explant by observing the effect of different concentrations of PGR on its growth.

2. METHOD & MATERIALS

2.1. Preparation of media

The preparation of media was adapted from Puad et al. (2016) [9]. For 1 L of medium, 4.4 g of MS powder was added. The Schott bottle was filled with 800 mL of distilled water. Then, according to the experimental design, 2,4-D was administered as the PGR with the concentrations of 5 mg/L, 10 mg/L, 15 mg/L, 20 mg/L and a control without 2,4-D, respectively. After that, 30 g/L sucrose, 50 mg/L casein hydrolysate and 7 g/L agar were added to the solution. The distilled water was added until it reached 1 L. The pH of the medium was adjusted to 5.8 by adding 1M of KOH or HCl. The medium was then sterilized for 15 minutes at 121°C.

2.2. Surface sterilization of cassava explants

The explants which are the leaves and stem were collected from cassava trees of Rayong cultivar that were planted at Mahallah Asiah compound, IIUM. The cassava leaves and stems were washed thoroughly under running tap water for about 30 minutes to clean any dust and contaminants on the surface after washing up using detergent [10]. Next, the explants were surface sterilized according to the experimental design using FCCCD by RSM in Design-Expert v9.0.6 (Tables 1 & 3). The chosen factors which are based on the literature were the time for the explants to be soaked in 70% ethanol (1, 3 and 5 min) and sodium hypochlorite (5, 10 and 15 min) as well as the concentration of sodium hypochlorite (1.5, 5.75 and 10 %). A number of 17 experiments were conducted with three replicates and the response was the percentage of contamination on explants (Eq. (1)) [11].

$$Percentage\ of\ contamination\ (\%) = \frac{Total\ number\ of\ contaminated\ explants}{Total\ number\ of\ explants} \times 100 \quad (1)$$

2.3. Preparation of PGR stock solution

The stock solution of 2,4-D was prepared according to Puad et al. (2016) [9]. About 100 mg of 2,4-D was added into a test tube. Then, 2 - 5 mL of ethanol or 1 M NaOH was added into the test tube to dissolve the powder. The test tube was heated gently if needed. When 2,4-D was completely dissolved, the solution was diluted by adding 100 mL of distilled water. The stock solution was then kept in the refrigerator until use. Eq. (2) is the general formula for preparation of PGRs stock solution [9].

$$Volume\ of\ Stock\ Solution = \frac{Desired\ Hormone\ Concentration \times Medium\ Volume}{Stock\ Solution\ Concentration} \quad (2)$$

2.4. Preparation of explants

The sterile stems were directly cut into 10-15 mm size. Meanwhile, for the sterile cassava leaves, their midvein were firstly removed before cutting them to about 10 mm X 10 mm size. Both explants were then placed onto the sterile filter paper to dry before culturing them into MS medium.

2.5. Initiation of explants

The leaves and stems were cultured onto the MS media in the petri dish. The explants were then incubated in a plant growth chamber at 26 ± 1˚C under the dark condition.

2.6. Effect of different 2,4-D concentrations on cassava callus culture initiation

After the optimization of surface sterilization condition for cassava explants, the stem explants were selected for callus initiation. The initiation of cassava callus culture was based on One Factor at Time (OFAT) approach. The concentrations of 2,4-D were varied (Table 6) while other culture conditions such as carbon source, type of medium and incubation condition were remained constant. The duration of the incubation period was 21 days (3 weeks) with the observation was recorded for every 3 days. All experiments were conducted in triplicates.

2.7. Callus growth analysis

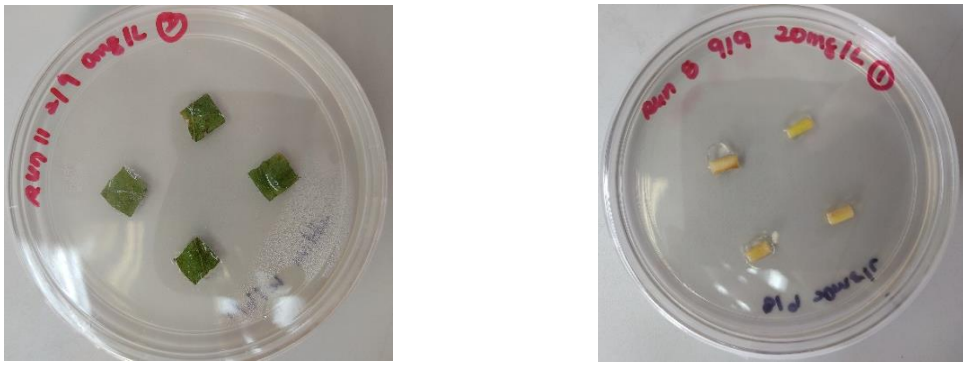
The callus growth was observed every 3 days to determine which day the callus started to emerge from the explants after inoculation. Then, after 3 weeks, the frequencies of callus formation was calculated by using Eq. (3) [8].

$$Frequencies\ of\ callus\ formation\ (\%) = \frac{Explants\ produce\ callus}{Total\ cultured\ explants} \times 100\% \quad (3)$$

3. RESULTS AND DISCUSSION

3.1. Optimization of surface sterilization condition for cassava explants

The collected explants which are cassava leaves and stems were cleaned with the detergent before being washed under running tap water for 30 minutes. Then, the explants were surface sterilized according to the design of the experiment shown in Table 1. After the explants were surface sterilized, the explants were then placed on the MS medium supplemented with 30 g/L sucrose and 50 mg/L casein hydrolysate (Fig.1). Each experimental runs have 3 replicates which contain 4 explants, and this gave a total of 12 explants for each run. The culture was observed every 3 days throughout the 21-days cultivation period for any contamination and data record.



(a) (b)

Fig. 1. Cassava leaf (a) and stem explant (b) inoculated on the MS medium.

3.1.1. Leaf explant

After three weeks of observation, the percentage of contamination were calculated and recorded (Table 1). The best cassava leaf surface sterilization method which gave the lowest percentage of contamination (16.67%) is Run 12 where the leaves were soaked for 10 min in 5.75% sodium hypochlorite after soaking the explants in 70% ethanol for 5 min. Then the second-lowest percentage of contamination is Run 7 where it gave 25% of contamination when the leaves were soaked in 70% ethanol for 3 min followed by 5.57% sodium hypochlorite for 15 min. Runs 3, 4, 5, 8 and 9 had their explants fully contaminated (100%). The 100% of the contamination that occurred in those runs might be due to the low concentration of sodium hypochlorite used which is 1.5% while for Run 5, the soaking time for 70% ethanol is not enough for the explants to prevent the microbial contamination. A longer handling time for leaf explants preparation after surface sterilization compared to the stems might also contribute to a high rate of contamination (Section 2.4).

Mahdi & Edward (2017) [12] conducted a study on *in vitro* propagation of *Putih* variety, one of the Malaysian casava varieties. The shoot tips were submerged in 70% ethanol for 1 min followed by 25% Clorox® (5.25% of sodium hypochlorite) added with 2 drops of Tween 20 for 10 min for surface sterilization procedure. Meanwhile, Okinyi (2012) [13] cut the leave explants (Kenya cassava varieties) into pieces before soaking them into 70% ethanol for 2 min, then transferred into 1.5% concentration of sodium hypochlorite for 15 min and lastly rinse 4 times with distilled water.

Table 1: The percentage of contamination of cassava leaf explants for each run based on FCCCD by RSM in Design-Expert v9.0.6

Run	Concentration of sodium hypochlorite (%)	Soaking time in sodium hypochlorite (min)	Soaking time in 70% ethanol (min)	Total number of contaminated explants	Total percentage of contamination (%)
1	10.00	15	1	11	91.67
2	10.00	15	5	6	50.00
3	1.50	5	5	12	100.00
4	1.50	5	1	12	100.00
5	5.75	10	1	12	100.00
6	5.75	10	3	7	58.33
7	5.75	15	3	3	25.00
8	1.50	10	3	12	100.00
9	1.50	15	1	12	100.00
10	10.00	10	3	9	75.00
11	10.00	5	1	9	75.00
12	5.75	10	5	2	16.67
13	1.50	15	5	11	91.67
14	10.00	5	5	10	83.33
15	5.75	5	3	10	83.33
16	5.75	10	3	4	33.33
17	5.75	10	3	4	33.33

Comparing between the literature and experimental results of this study, the surface sterilization method used did not differed significantly in terms of soaking time in 70% ethanol and sodium hypochlorite as well as its concentration. Table 2 sumarizes the comparative review on surface sterilization techniques for cassava explants, specifically for its leaf. There is no comparison on the percentage of contamination from the other 2 studies (Table 2) since the focus of these studies were not on the optimization of surface sterilization techniques for cassava explants. To our best knowledge, so far there is no paper on optimizing surface sterilization techniques for cassava explants. Comparison made in Table 2 is merely to compare the condition used for cassava explant surface sterilization method and not for the percentage of contamination.

Table 2: Comparative review on surface sterilization techniques for cassava explants

Explants	Concentration of sodium hypochlorite (%)	Soaking time for 70% ethanol (min)	Soaking time for sodium hypochlorite (min)	Reference
Leaf	5.75	5	10	This study
Shoot tips	5.25	1	10	(Mahdi & Edward, 2017)
Leaf	1.5	2	15	(Okinyi, 2012)

The results in Table 1 were then further analyzed by using ANOVA in Design-Expert v9.0.6. However, the *p-value* for the model is more than 0.05 which indicates that the model is not significant. The significant model term is A^2 while the other terms fall under not significant. The lack of fit is not significant as the *p-value* is more than 0.1. The R^2 value is 0.7010, which showed that half the degree correlation between the experimental and

predicted values. Meanwhile, the predicted R^2 is -1.3861 and the adjusted R^2 is 0.3165. The value is not acceptable because the difference between the adjusted R^2 and predicted R^2 is more than 0.2, thus indicate that the model is not significant and has a lower degree of significant. Due to this, the regression equation as well as 3D-surface plot are not included for the analysis and discussion.

3.1.2. Stem explant

The same procedures were carried out for the cassava stems. After three weeks of observation, the percentage of contamination were calculated and recorded (Table 3).

Table 3: The percentage of contamination of cassava stem explants based on FCCCD by RSM in Design-Expert v9.0.6

Run	Concentration of sodium hypochlorite (%)	Soaking time in sodium hypochlorite (min)	Soaking time in 70% ethanol (min)	Total number of contaminated explants	Percentage of contamination (%)
1	10.00	15	1	10	83.33
2	10.00	15	5	11	91.67
3	1.50	5	5	12	100.00
4	1.50	5	1	12	100.00
5	5.75	10	1	4	33.33
6	5.75	10	3	7	58.33
7	5.75	15	3	9	75.00
8	1.50	10	3	12	100.00
9	1.50	15	1	12	100.00
10	10.00	10	3	12	100.00
11	10.00	5	1	12	100.00
12	5.75	10	5	3	25.00
13	1.50	15	5	12	100.00
14	10.00	5	5	12	100.00
15	5.75	5	3	12	100.00
16	5.75	10	3	8	66.67
17	5.75	10	3	9	75.00

Based on Table 3, it can be concluded that the best cassava stem surface sterilization method which achieved the lowest percentage of contamination is Run 12 where the leaves were soaked for 10 min in 5.75% sodium hypochlorite after soaking the explants in the 70% ethanol for 5 min. Then the second lowest percentage of contamination is Run 5 where it gave 33.33% of explants contaminated when the stems were soaked in 70% ethanol for 1 min followed by 5.57% sodium hypochlorite for 10 min. Runs 3, 4, 8, 9, 10, 11, 13, 14 and 15 gave 100% of contamination. The 100% of contamination occurred in Runs 3, 4, 8, 9, and 13 is caused by the low concentration of sodium hypochlorite used which is 1.5% while for Runs 10, 11 and 14, the concentration for sodium hypochlorite (10%) and the soaking time might be too high for the explants to survive [14]. For Run 15, the soaking time of stem explants in 70% ethanol and sodium hypochlorite, respectively were too short to prevent contamination.

The result from this study is almost similar with Maruthi et al. (2019) [15], which used 5% sodium hypochlorite for the cassava stems in generating virus-free cassava plants due to viral disease epidemic in Africa. Other than that, Ogero (2012) [16] soaked the cassava

stem explants in 70% ethanol for 2 minutes followed by the Jik (bleach) for 15 min. Similarly, Okinyi (2012) [13] soaked the cassava stem explants into 70% ethanol for 2 min before transferring into 1.5% concentration of sodium hypochlorite for 15 min and finally rinsed for 4 times with sterile distilled water. In this study, since calli were successfully induced from the stem part of cassava compared to its leaves (Section 3.1.1), only stem was used as the explant for the next objective.

Comparing between the literature and experimental results of this study, the surface sterilization procedure did not differ significantly in terms of soaking time in 70% ethanol and sodium hypochlorite as well as its concentration. Based on the optimization result, cassava stem explant should be soaked in 5.75% of sodium hypochlorite for 10 minutes after the explants were dipped in 70% ethanol for 5 minutes. Table 4 depicts the comparative review on surface sterilization techniques for cassava explants, specifically its stems. There is no comparison on the percentage of contamination made in Table 4 since these 2 studies did not reported on surface sterilization techniques for cassava explants. From the literature review, so far there is no paper on optimization of surface sterilization techniques for cassava explants. Thus, Table 4 is only to compare the condition used for the surface sterilization method and not for the percentage of contamination.

Table 4: Comparative review on surface sterilization techniques for cassava stem explants

Explants	Concentration of sodium hypochlorite (%)	Soaking time for 70% ethanol	Soaking time for sodium hypochlorite (min)	Reference
Stem	5	3-5 sec	20 -30	(Maruthi et al., 2019)
Stem	1.5	2 min	15	(Okinyi, 2012)
Stem	5.75	5 min	10	This study

The ANOVA analysis for the model on stem experiment is concluded in Table 5. Based on Table 5, *p-value* for the model is less than 0.05 which indicates that the model is significant. The significant model terms are A^2, B^2 and C^2 while the other terms fall under not significant. The lack of fit is not significant as the *p-value* is more than 0.1 which is 0.6771. The R^2 value is 0.9598, which shows that a higher degree correlation between the experimental and predicted values. It is best to have a higher R^2 compared to a lower R^2 since a higher R^2 value reflects that the model fits the data better [17]. The predicted R^2 is 0.7838 and the adjusted R^2 is 0.9082. The value is acceptable because the difference between the adjusted R^2 and predicted R^2 is less than 0.2. It can be concluded that the model is significant and has a higher degree of significant.

Fig. 2, 3 and 4 represent the 3D graph for the response of percentage of contamination with different parameters. Fig. 2 shows the upward curvature effect for the quadratic response surface plot of concentration of sodium hypochlorite and time for sodium hypochlorite with a constant time for ethanol, 3 min. It illustrates the minimization of percentage of contamination at the middle of the graph based on the concentration and soaking time for sodium hypochlorite. The predicted lowest percentage of contamination from the plot (Fig. 2) is located at the middle where the sodium concentration is 5.75% and the soaking time for sodium hypochlorite is 10 min. A lower concentration of sodium hypochlorite (10%) and shorter soaking time for sodium hypochlorite (5 – 10 min) would increase the percentage of contamination. Exposure of explants towards shorter soaking time with lower concentration may cause incomplete sterility of the explants to initiate callus culture. This also applied to the higher concentration of sodium hypochlorite (1.5%)

and higher soaking time of sodium hypochlorite (5 – 10 min). Longer exposure of soaking time with higher concentration of sodium hypochlorite may cause the explants to turn brown and eventually died.

Table 5: ANOVA analysis on the cassava stem explants

Source	Sum of squares	Degree of freedom	Mean square	F value	*p-value*	
Model	0.91	9	0.10	18.58	0.0004	Significant
A - Concentration of Sodium hypochlorite (%)	6.250 E-003	1	6.25 E-003	1.15	0.3200	
B – Soaking time in sodium hypochlorite (min)	0.025	1	0.025	4.58	0.0696	
C – Soaking time in 70% ethanol (min)	2.779 E-003	1	2.779 E-003	0.51	0.4986	
A^2	0.40	1	0.40	73.41	<0.0001	
B^2	0.18	1	0.18	33.62	0.0007	
C^2	0.28	1	0.28	50.80	0.0002	
AB	7.813 E -003	1	7.813 E-003	1.43	0.2704	
AC	8.694 E-004	1	8.694 E-004	0.16	0.7017	
BC	8.694 E-004	1	8.694 E-004	0.16	0.7017	
Residual	0.038	7	5.457 E-003			
Lack of fit	0.024	5	4.861 E-003	0.70	0.6771	Not significant
Pure error	0.014	2	6.947 E-003			
Cor Total	0.95	16				

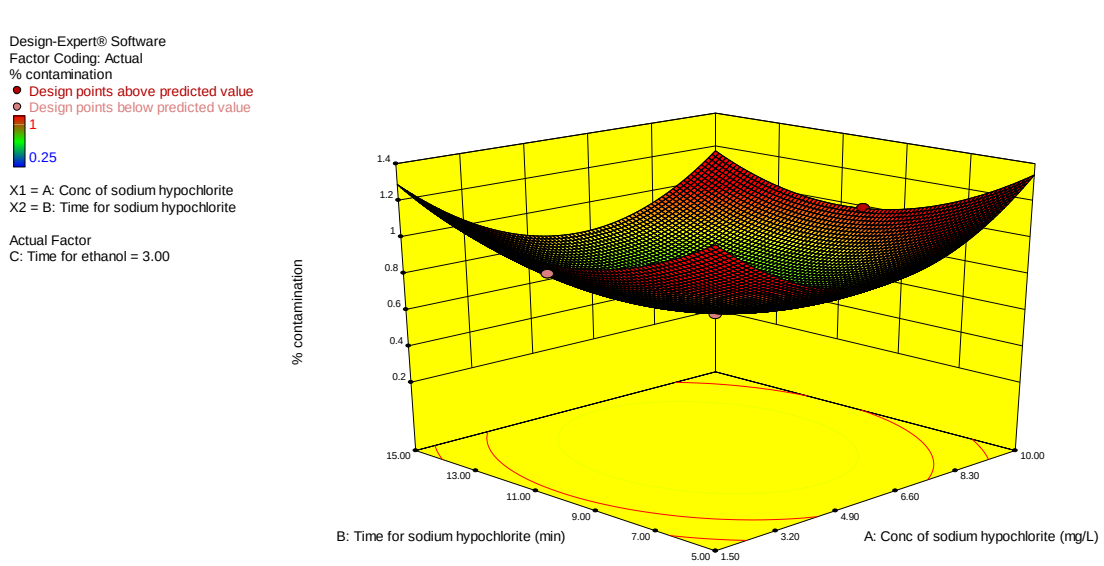


Fig. 2. 3D surface of percentage of contamination based on the interaction of sodium hypochlorite concentration and soaking time in sodium hypochlorite from FCCCD.

The plot in Fig. 3 is a graph of percentage of contamination depending on the soaking time in 70% ethanol and concentration of sodium hypochlorite. The highest percentage of contamination occurred when the soaking time for 70% ethanol is at 3 min for 1.5% and 10% concentration of sodium hypochlorite while for the lowest percentage of contamination occur at 5 min soaking time in 70% ethanol and 5.75% concentration of sodium hypochlorite. Meanwhile, the plot in Fig. 4 is a graph of percentage of contamination dependent on the soaking time for 70% ethanol and sodium hypochlorite, respectively. The constant for the plot is the concentration of sodium hypochlorite which was at 5.75%.

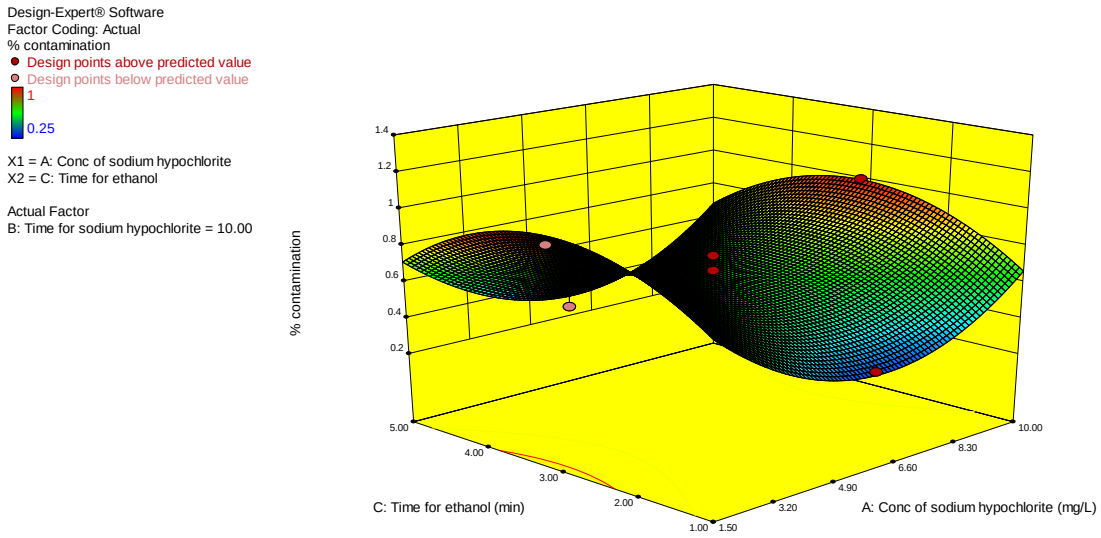


Fig. 3. 3D graph of percentage of contamination based on the interaction of sodium hypochlorite concentration and soaking time in 70% ethanol from Design-Expert v9.0.6.

Based on Fig. 4, at 5.75% concentration of sodium hypochlorite with a soaking time 5 minutes in 70% ethanol and 10 minutes for sodium hypochlorite, the percentage of contamination is the lowest. When the concentration of sodium hypochlorite used is higher, the soaking time of sodium hypochlorite should be lower, and the same applied for soaking time in 70% ethanol. This is because a longer exposure in a high concentration of sodium hypochlorite will be harmful to the explants as observed by Kuppusamy et al. (2019) [8]. The explants of Eucalyptus hybrid species turned into brown and eventually died due to a longer exposure of soaking time in $NaOCl_2$.

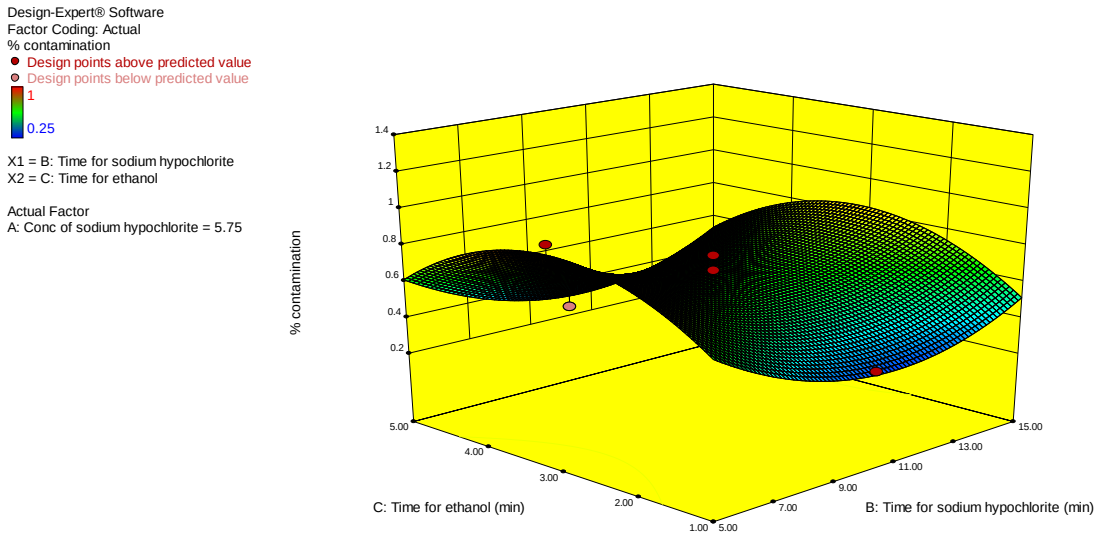


Fig. 4. 3D graph of percentage of contamination based on the interaction of soaking time in sodium hypochlorite and soaking time in 70% ethanol from Design-Expert v9.0.6.

3.2. Initiation of *Manihot esculenta* Crantz callus culture

Based on the result in Section 3.1.2, stem was used for the initiation of cassava callus culture. The procedure for stem surface sterilization was conducted based on the optimized condition. The effect of different concentrations of 2,4-D (0, 5, 10, 15, 20 mg/L) as the PGR to initiate cassava callus culture was investigated using one factor at a time (OFAT) approach. Each concentration of 2,4-D was conducted in 3 replicates containing 4 stem explants. The observation was made every 3 days for 21-days of incubation period. The callus started to emerge from the stem explant on Day 9. On Day 15, the growth of callus was clearly observed (Fig 5). The callus was left to grow and observed every 3 days until Day 21. Since the size of callus grown was small to be harvested and weighed, the callus was subcultured to a new media with the same concentration of 2,4-D to allow further growth.

Fig. 5. The growth of callus on Day 15.

Table 6 shows the frequency of callus formation for different concentrations of 2,4–D. The frequency of callus formation was calculated using Eq. (3).

Table 6: The frequency of cassava callus formation for different 2,4-D concentrations

No	Concentration of 2,4–D (mg/L)	No of callus grown	Frequency of callus formation (%)
1	0	1	8.33
2	5	5	41.67
3	10	4	33.30
4	15	4	33.30
5	20	2	16.67

The highest frequency of callus formation was on the media with 5 mg/L 2,4-D (41.67%) while the lowest frequency of callus formation (8.33%) was achieved when the media is not supplemented with 2,4-D (Table 6). According to Taha et al. (2013) [6], induction of callus generally requires an auxin for the explants cell to revert to a dedifferentiated state and begin to divide. The most frequent auxin that has been used to induce callus culture is 2,4-D [8]. Based on Table 6, both callus formation frequency for media with the concentration of 10

and 15 mg/L of 2,4-D achieved the same result which is 33.33%. The media with 20 mg/L of 2,4-D gave 16.67% frequency of callus formation. Based on the results, a higher concentration of 2,4-D supplied to the medium gave a lower frequency of cassava callus formation. The results of this study contradicted with the ones reported by Taha et al. (2013) [6]. It is concluded that MS medium supplemented with 15 mg/L 2,4- D yielded the highest callus formation of 74.9% using cassava leaves as the explant.

In addition, according to Taha et al. (2013) [6], the stem explant was the second-best explants after the leaves with 66.6% of frequency of callus formation and root was the least (29.1%). Furthermore, no response for callus induction was observed when no PGR was added for all types of explants [6]. The differences mentioned above might be due to different cultivars of cassava used.

Meanwhile, Fletcher et al. (2011) [18] investigated the effect of 2,4-D on callus induction using different cassava explants and Ghana cultivar types. For the three different auxin concentrations used (8, 12 and 15 mg/L 2,4-D), the percentage of callus production ranged from 0 to 75 % for different explants such as leaves, bark, and buds. The largest proportion per explants were obtained from the bark of *Afisiafi* cultivar grown in a media with 12 mg/L 2,4-D. For cultivar *Tuaka,* the percentage of callus formed from the explants such as leaf, stem petiole, and buds did not change significantly at any of the three 2,4-D concentrations. In general, a higher frequency of callus formation was obtained from the leaf explant and the least was achieved by the bark explants. Different types of explant of *Afisiafi* cultivar also gave differences in the percentage of callus formed where the leaf explants achieved the best results compared to petiole and buds, respectively in the media supplemented with 8 mg/L 2,4-D. For *Afebankye* and *Doku* cultivars, varying 2,4-D concentrations produced the same quantity of callus from leaf, petiole, and buds explants [18].

Based on the literature review and results of this study, it can be concluded that different cassava varieties or cultivars and types of explants give different responses towards different concentrations of supplied PGR, specifically 2,4-D in the initiation of callus culture. This is due to different levels of totipotency of different cultivars [18]. Table 7 summarizes the comparative review on the effect of 2,4-D for casava callus culture initiation.

Table 7: The comparative review of effect of 2,4-D on initiation callus culture.

Cultivar	Explant type	Concentration of 2,4-D (mg/L)	Percentage of callus formation (%)	Reference
Rayong	Stem	5	41.67	This study
Not available	Stem	15	66.6	[8]
	Root	15	29.1	
Doku	Leaf	8 & 15	40	[18]
	Leaf	8	65	
Afisiafi	Bark	12	55	
Afebankye	Leaf	8	75	
	Stem petiole	12	45	
Tuaka	Leaf	8	14	
	Stem petiole	15	20	

4. CONCLUSION

For cassava cultivar type Rayong, the best surface sterilization procedure is to expose the explants for 5 minutes in 70% ethanol, followed by soaking them in 5.75% sodium

hypochlorite for 10 minutes. Both leaf and stem explants achieved the lowest percentage of contamination for the above condition with 25% and 16.67%, respectively. 2,4-D at the concentration of 5 mg/L gave the highest frequency of callus formation which is 41.67% followed by 10 mg/L (33.3%), 15 mg/L (33.33%) and 20 mg/L (16.67%). It can be deduced that different cultivars of cassava explants respond differently towards different types and concentrations of PGRs used for callus formation. Thus, optimization studies on different cassava cultivars for the types of explant, PGRs and their concentration is highy recommended for maximum callus growth. The findings from this study can be further explored in the future to utilize cassava plant culture for starch production.

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