https://doi.org/10.31436/iiumej.v25i1.2585

THE INFLUENCE OF PLANT GROWTH REGULATORS AND LIGHT SUPPLY ON BITTER CASSAVA CALLUS INITIATION FOR STARCH PRODUCTION

NOOR ILLI MOHAMAD PUAD[1*], SOUMAYAT ALI IBRAHIM MZE[1], AZLIN SUHAIDA AZMI[1] AND MUHAMMAD YUSUF ABDUH[2]

[1]Department of Chemical Engineering and Sustainability, Kulliyyah of Engineering, International Islamic University Malaysia, Kuala Lumpur, Malaysia
[2]School of Life Sciences and Technology, Institut Teknologi Bandung, Jalan Ganesha, No. 10, Bandung, 40132 Indonesia

[*]Corresponding author: illi@iium.edu.my

(Received: 26 August 2022; Accepted: 26 June 2023; Published on-line: 22 December 2023)

ABSTRACT: The application of cassava starch in the biopolymers industry has been growing significantly due to its low cost, good oxygen barrier in the dry state, and biodegradability. Plant culture technology is an alternative to traditional propagation as it does not require large areas for production, has a higher rate of multiplication, and produces disease-free plants. However, the application of cassava callus culture for starch production is limited. This study focuses on identifying the significant culture parameters for a maximum Rayong cultivar cassava callus growth and evaluation of its starch content. Cassava stems petiole and leaf explants were cultured on MS medium containing different combinations of 2,4-D (8, 12, and 15 mg/L) and BAP (1, 3, and 5 mg/L) under three light conditions (0, 16, and 24 h). The screening of the most influential parameter was done using the 2-level Factorial Design in Design Expert v13 by analyzing the frequency of callus formation. All leaf explant turned brown with no callus induction. The highest frequency of callus formation derived from stem petiole explant was achieved by the combination of 8 mg/L 2,4-D and 1 mg/L BAP under the light condition (75%) followed by 8 mg/L 2,4 D + 1 mg/L BAP under the dark condition (50%). Based on the ANOVA analysis, the individual supply of 2,4-D and BAP respectively, have a negative effect on callus formation while the combination of 2,4-D and BAP has a positive effect. Light supply did not significantly affect cassava callus formation. The amount of starch in the cassava callus was then investigated using an iodine test which yielded 0.21% of the total weight of the callus (0.0101g). The amount of starch is relatively low considering that the callus was not grown under the optimum condition for starch production. The findings of this study open prospects for future research in cassava cultures in favor of starch production.

ABSTRAK: Penggunaan kanji ubi kayu dalam industri biopolimer telah meningkat secara mendadak disebabkan oleh faktor kosnya yang rendah, rintangan oksigen yang baik dan sifat keterbiodegradasi. Teknologi kultur tisu tumbuhan merupakan alternatif kepada pembiakan secara tradisional yang mana teknologi ini tidak memerlukan kawasan penanaman yang luas, penghasilan yang lebih singkat dan menghasilkan tumbuhan bebas penyakit. Walau bagaimanapun, kajian mengenai penggunaan kanji dari kultur ubi kayu masih belum meluas. Kajian ini bertujuan mengenal pasti parameter penting dalam pertumbuhan maksimum kalus kultivar ubi kayu Rayong dan kandungan kanjinya. Eksplan dari tangkai petiol dan daun ubi kayu dikulturkan dalam medium MS yang mengandungi pelbagai kombinasi hormon tumbuhan 2,4-D (8, 12 dan 15 mg/L) dan BAP (1, 3 dan 5 mg/L) di bawah tiga tempoh masa pencahayaan (0, 16 dan 24 jam). Saringan parameter paling berpengaruh dilakukan menerusi reka bentuk faktorial 2-peringkat perisian Design Expert v13 melalui analisa frekuensi

pembentukan kalus. Semua eksplan daun telah bertukar dari hijau ke perang dan tidak menunjukkan induksi kalus. Bagi eksplan batang daun, frekuensi pembentukan kalus tertinggi diperoleh dari kombinasi 8 mg/L 2,4-D dan 1 mg/L BAP di bawah pencahayaan 24 jam (75%) diikuti dengan 8 mg/L 2,4 D + 1 mg/L BAP di bawah pencahayaan malap (50%). Berdasarkan analisis ANOVA, medium 2,4-D dan BAP masing-masing menunjukkan kesan negatif kepada pembentukan kalus, sementara kombinasi 2,4-D dan BAP memberikan kesan positif. Dapatan ini menunjukkan bahawa pencahayaan tidak signifikan terhadap pembentukan kalus kultur ubi kayu. Kandungan kanji di dalam kalus ubi kayu kemudiannya diuji dengan larutan iodin, dan menunjukkan dapatan sebanyak 0.21% dari berat kalus (0.0101 g). Secara relatif, nilai ini adalah rendah memandangkan eksperimen ini bukan dihasilkan di bawah keadaan optimum bagi pembentukan kanji. Hasil kajian ini membuka peluang kepada kajian-kajian lain di masa hadapan dalam penghasilan kanji dari kultur tisu ubi kayu.

KEYWORDS: *cassava; callus culture; 2,4-dichlorophenoxyacetic acid (2,4-D); 6-benzylaminopurine (BAP); light*

1. INTRODUCTION

The application of cassava (*Manihot esculenta* Crantz) starch in the biopolymers industry has been growing significantly due to its availability (particularly in seed and tuber plants), low-cost, good oxygen barrier in the dry state, renewability, and biodegradability [1]. Cassava starch has also gained large popularity as a starch source because of its low gelatinization temperature, high binding capacity, and viscosity [2]. For the manufacturing of a sustainable and efficient bioproduct raw material, bitter cassava is preferred over sweet cassava as it has high starch content, high production yield, and high chemical defense. The exploitation of 30% of waste from bitter cassava can be self-sufficient as a feedstock requirement for food, medical and packaging industries [3]. In accordance with that, it is estimated that the global cassava starch market will reach USD 8.1 billion in the next five years (2023-2027) [4].

However, cassava starch usage is facing some limitations in terms of mass propagation. The increase in demand requires several thousand hectares of arable land, which is limited since there is a decline in empty lands due to economic development. Moreover, traditional cassava cultivation records high pest and disease invasions, and requires long periods of cultivation [5,6]. Therefore, as an alternative, plant tissue culture has been used as a substitute for traditional propagation as it has been proven to remediate insufficient propagation and provide disease-free cassava plants. Additionally, *in vitro* cultivation is not dependent on geographical, seasonal, and environmental variations as it controls the plant's environment (nutrient media, physical factors, growth hormones, and aseptic condition) [7].

Among different types of plant tissue culture, callus culture is the most widely used due to its pluripotency. Although this culture is comprised of cells that have no specific function, this culture plays an important role in plant culture technology as it is being used as the starter for other cultures. For instance, this culture is used to initiate cell suspension culture which is frequently used in plant transformation studies or large-scale secondary metabolite production. Moreover, callus culture is also very crucial in the regeneration of plants as well as plant organ production. Callus culture can regenerate any part of the plant with the use of a suitable plant growth hormone [8]. The established conditions such as nutrient media and plant growth regulators (PGRs) influence highly on the callus culture induction and growth.

To our best knowledge, reports on cassava callus culture for starch production are still scarce. Most studies aimed to provide an effective callus induction based on the effect of different PGRs as well as alternative genetic modification to provide rapid production of the

cassava plant [8-10]. Moreover, no report is yet available specifically for the Rayong cultivar which was used in this study. Rayong cultivar is a bitter type of cassava, with a high starch content, wide adaptability to different environmental conditions, and high yield potential [11]. Thus, this study aimed to identify the effect of PGRs and light supply on the initiation of Rayong cultivar callus culture and preliminary evaluation of its starch content.

Plant growth regulators (PGRs) are hormones that affect the growth and development of a plant. In the case of callus growth, the major PGRs that influence its development are auxin and cytokinin. 2,4-dichlorophenoxyacetic acid (2,4-D) has been reported as the most effective auxin for cassava callus formation [8,12]. For cytokinin, thidiazuron (TDZ) induced a higher percentage of callus formation followed by 6-Benzylaminopurine (BAP) and kinetin. It is concluded that TZD induced the formation of indole-3-acetic acid (IAA) in the cell which helps callus formation [9]. However, whether the combination of auxin and cytokinin on cassava callus initiation and growth might have a positive impact has yet to be reported. Therefore, this study explored the effect of combining 2,4-D (8, 12, and 15 mg/L) and BAP (1, 3, and 5 mg/L) on cassava callus initiation.

Moreover, light supply, as another culture parameter, was also investigated. Cassava callus culture can grow in both dark and light conditions, at a temperature of 25-28 °C within 1 month [8-9,12]. Light supply influenced the initiation time of the culture *i.e.* white-light exposure culture initiates faster callus formation than the dark culture. Accordingly, continuous light, 16-h light, and continuous dark conditions were chosen as the range for the light supply parameter. Cassava callus culture was initiated according to these parameters with constant monitoring of the process for 30 days with an interval of 3 days of sampling. A 2-level factorial design in Design Expert v13 (Stat-Ease, Inc., USA) was used for the experimental design and screening purposes. Finally, an iodine test was conducted for the preliminary evaluation of starch amount in the induced cassava callus.

2. MATERIALS AND METHODS

2.1 Materials

The explants (leaves and stem petiole) were obtained from the cassava trees (Rayong cultivar) planted at International Islamic University Malaysia. Ethanol was purchased from HmbG Chemicals, Germany while sodium hypochlorite was purchased from Bendosen, Malaysia. Both TWEEN® 20 and Gelrite were purchased from Sigma-Aldrich, UK. Murashige and Skoog (MS) basal medium, sucrose, casein hydrolysate were purchased from Duchefa, Netherlands. Meanwhile, hydrochloric acid (HCl), 2,4-D, and BAP were purchased from R&M Chemicals, UK. Sodium hydroxide (NaOH) was from Merck, UK.

2.2 Surface Sterilization of Cassava Explants

The surface sterilization procedure was adapted and modified based on Puad et al. [13]. Leaves and stem petioles of the Rayong cultivars were washed with antibacterial detergent for 5 min followed by washing with 50% thiram solution under continuous shaking for 1 hour. Each washing was followed by 3 rinses of distilled water. Next, the explants were soaked in 70% ethanol for 5 min, then bleached with 5.75% sodium hypochlorite with 2-3 drops of TWEEN® 20 for 10 min. After each soaking, the explants were rinsed at least three times with sterile distilled water [9,12]. The sterile explants were cut into small pieces of 5 mm which were inoculated in a media plate (4 explants per plate).

https://doi.org/10.31436/iiumej.v25i1.2585

2.3 Preparation of Culture Media

The media preparation used was done following the method proposed by Puad & Tang [14]. The media contained 4.4 g/L of MS basal medium, 30 g/L of sucrose, 0.05 g/L of casein hydrolysate, 2.5 g/L of Gelrite, and 0.75 mL of plant protection mixture (PPM). The media was adjusted to a pH of 5.7 using 1 M of NaOH and 1 M of HCl and sorted following different combinations of 2,4-D and BAP concentration as presented in Table 1.

Table 1: Combinations of different concentrations of 2,4-D and BAP used in the study

No.	Combinations of 2,4-D: BAP (mg/L)
1	8:1
2	8:5
3	12:3
4	15:1
5	15:5

Then, the media were autoclaved at 121°C, 15 psi for 20 min and let to cool down till they reached 40 °C. The sterile explants were placed on the media, in sterile conditions and the cultures were incubated under three light conditions which are continuous light supply (24 h), 16-h light exposure, and continuous dark condition (0 h). The frequency of callus formation was the response used to evaluate the rate of callus formation during a period of 30 days.

2.4 Experimental Design

Using Design Expert v13, a 2-level factorial design was set up based on 3 different factors which are 2,4-D (8, 12, 15 mg/L), BAP (1, 3, 5 mg/L), and light exposure period (0-24 h) using cool-white fluorescent tube lights (2500 lm) resulting in 27 runs. The design was replicated two times for two different explants (cassava leaf and stem petiole). Each experiment was conducted in triplicate. Table 2 presents the design summary of the experiment. The effect of three factors was tested through the ANOVA analysis of the frequency of callus formation.

Table 2: The range of three factors for 2-level factorial design experiments

Factor	Name	Units	Low	Middle	High
A	2,4 D	mg/L	8	12	15
B	BAP	mg/L	1	3	5
C	LIGHT	h	0	16	24
Response	Frequency of callus formation	%			

2.5 Analytical Procedures

2.5.1 Callus Growth

During the 30 days of the incubation period, the observation on the frequency of callus formation was recorded every 3 days. The frequency of cassava callus formation was calculated using Eq. (1):

$$Frequency\ of\ callus\ formation(\%) = \frac{Explants\ produced\ callus}{Total\ cultured\ explants} \times 100 \quad (1)$$

https://doi.org/10.31436/iiumej.v25i1.2585

2.5.2 Starch Content in Callus

The extraction of starch from the 0.0102 g of callus obtained from the initiation of the cassava callus culture was done following the method described in Reddy & Bhotmange [15]. The callus was dried in an oven (Memmert GmbH, Germany) for 48 h at 70 °C and then finely ground using a mortar and pestle. The protein molecules were removed by dispersing the callus in 0.153 mL of distilled water. The solution was adjusted to a pH of 10 by adding a few drops of NaOH and mixing moderately for 1 hour. Then, the starch was extracted by 3 sequential centrifugations at 5000 rpm for 30 min each. For each centrifugation, the supernatant was discarded, and the pellets were treated with 0.0306 mL of NaOH. The starch was resuspended in a mixture of 90% ethanol and NaOH for 1 hour at 80°C and let to cool to room temperature before adding distilled water to a volume of 10 mL. This last step was also performed with 0.10 g of commercial starch for the preparation of the standard starch solution.

To measure the starch content of the callus, a standard curve was constructed using different concentrations of commercial starch solution *i.e.,* 8, 16, 24, 32, 40%. The absorbance at 620 nm was recorded. Eq. (2) was generated from the starch standard curve where Y is the absorbance of starch solution and X is the percentage of starch standard solution (%). Eq. (2) was then used to calculate the percentage of starch in the sample solution before the amount of starch (g) in the callus was determined using Eq. (3).

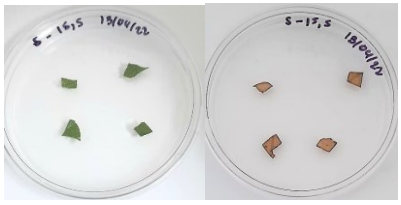
$$Y = 0.0138X + 0.0179 \tag{2}$$

$$Amount\ of\ starch\ (g) = \frac{X \times iniatial\ amount\ of\ callus}{100} \tag{3}$$

3. RESULTS AND DISCUSSION

3.1 Callus Induction from Cassava Leaf Explants

After 1 month, it was observed that there were no calluses induced for any types of PGRs and concentrations derived from the cassava leaf explants. Instead, the leaf explants turned brown, and no response was observed (no callus initiation, no contamination) indicating that the explants were dead (Fig. 1). This could have been caused by the fact that the explants were soaked in 70% ethanol for 5 min during the sterilization of the explants. Indeed, ethanol is an excellent sterilization chemical, yet it is extremely phytotoxic to the plant cells when exposed for a long period time. According to Bello et al. [16], exposing *Solnacio biafrae* explant with ethanol for 20 s gave a high survival rate compared to 3 min and 5 min, respectively. The exposition period of the explant to ethanol is different for each plant and from different parts of the same plant [17]. As an example, leaf cells have less lignified thin cell walls except the leaf epidermis [12]. The thinness of the leaf cell wall might be one of the reasons why the leaf explant could not withstand our sterilization technique. Thus, to obtain a 'clean and live' leaf explant, further study on the sterilization of each type of explant needs to be developed and taken account to avoid loss of the explants, time, and material.



(a) (b)

Fig. 1: Initiation of cassava callus on MS medium from leaf explant. (a) Day 1, (b) Day 30.

https://doi.org/10.31436/iiumej.v25i1.2585

3.1 Callus Induction from Cassava Stem Petiole Explants

3.1.1 Frequency of Callus Formation

Data tabulated in Table 3 clearly shows that callus was successfully induced from the combination of the first combination (8 mg/L of 2,4-D and 1 mg/L BAP) in both light conditions (75% to 0%) and dark conditions (50%) while explants placed in 12 mg/L 2,4-D and 3 mg/L BAP; 15 mg/L 2,4-D and 1 mg/L BAP; and 15 mg/L 2,4-D and 1 mg/L BAP induced very little callus (25%) to no callus induction (0%), respectively. Table 3 also demonstrates that the callus induction occurred from day 12 to day 24 in which faster growth was observed under the continuous light condition (24 h) compared to the dark condition (0 h). These results indicated that the combination and concentration of 2,4-D and BAP, as well as the light conditions, have an influence on the cassava callus growth and a lower concentration of auxin (2,4-D) and cytokinin (BAP) (8 mg/L 2,4-D + 1 mg/L) is favorable for cassava callus initiation.

Table 3: Summary of cassava callus initiation with different concentrations of 2, 4–D and BAP, and light conditions

Run	Factor A: 2,4D [mg/L]	Factor B: BAP [mg/L]	Factor C: LIGHT [h]	Callus induction [day]	Response: Frequency of callus formation [%]
1	8	5	0	24	25
2	15	5	24	-	0
3	8	1	24	14	50
4	8	5	24	-	0
5	15	5	0	-	0
6	15	1	0	-	0
7	15	1	24	-	0
8	8	1	0	14	25
9	12	3	16	24	25
10	8	5	24	27	25
11	8	1	0	24	50
12	15	5	0	-	0
13	15	5	24	-	0
14	8	1	24	16	50
15	15	1	24	-	0
16	8	5	0	24	50
17	15	1	0	-	0
18	12	3	16	-	0
19	8	5	24	-	0
20	8	1	0	24	50
21	15	5	24	-	0
22	15	5	0	-	0
23	8	5	0	-	0
24	12	3	16	-	0
25	8	1	24	12	75
26	15	1	0	-	0
27	15	1	24	-	0

https://doi.org/10.31436/iiumej.v25i1.2585

3.1.2 ANOVA analysis

The ANOVA analysis of the stem petiole shows that the *p-value* for the model was less than 0.05, and the F-value was 28.51 which indicates that the model was significant (Table 4). The significant model terms were factors *A, B* and *AB*. Factor C (light condition) was part of the residuals since it is less significant (no effect on the model). Moreover, the difference between the adjusted R^2 and the predicted R^2 was less than 0.2 and R^2 was 0.8029 which was a higher value indicating a high degree of correlation between the experimental and predicted data. Hence it could be deduced that the model has a high degree of significance [18]. The equation of the design as shown in Eq. (4) suggests that a lower value of factor A and B, respectively results in a higher frequency of callus formation while a higher value of factor AB will give a higher frequency of callus formation.

Table 4: ANOVA analysis of cassava callus growth from the stem explants

Source	Sum of Squares	Degree of freedom	Mean square	*F-value*	*p-value*	
Model	10458.73	4	2614.68	28.51	<0.0001	Significant
***A*- 2,4-D concentration (mg/L)**	5162.14	1	5162.14	42.58	<0.0001	
***B*- BAP concentration (mg/L)**	2604.17	1	2604.17	21.48	0.0001	
AB	2604.17	1	2604.17	21.48	0.0001	
Residual	2546.19	21	121.25			
Lack of fit						Not significant
Pure error						
Cor Total	12916.67	26				

R^2 = 0.8029, Adj – R^2 = 0.7747, Pred – R^2 = 0.6771

$$Frequency\ of\ callus\ formation\ (\%) = +129.21919 - 8.64981A - 22.32143B + 1.48810AB \quad (4)$$

The results of this study opposed the previous studies which reported that a higher concentration of auxin and cytokinin is favorable for callus growth while a lower concentration of auxin and cytokinin favors organ culture growth [8,9]. However, the BAP concentration range used in this study (1 mg/L to 5 mg/L) is not within the BAP concentration range (0.1 mg/L to 1 mg/L) used in Faye et al. [9], which may cause the differences. The contradiction of the results obtained could also be due to the cultivar type and explant used. Fletcher et al. [12] proposed that different explants and cultivars react differently to different concentrations of PGR. In their study using different cassava cultivars available in Ghana, among the 3 concentrations of 2,4-D used *i.e.,* 8, 12, and 15 mg/L, 2,4-D at 8 mg/L achieved a better callus growth of *Afisiafi* cultivar for both leaf (75%) and bark (25%). In addition, for the *Afebankye* cultivar, the leaf explant gave a better result for 8 mg/L 2,4-D (75%) while the stem petiole supplemented with 12 mg/L 2,4-D achieved 45% of callus formation. However, for *Tuaka* cultivars the percentage of formed callus from the explants such as leaf, stem petiole, and buds did not change significantly at any of the three 2, 4-D concentrations (below 20%). Based on this, it can be proposed that the stem petiole explant from the Rayong cultivar resulted in better callus growth at a lower concentration of 2,4-D (8 mg/L).

Besides that, this study explored the effect of combining the 2,4-D and BAP at various concentrations on cassava callus initiation which so far has not been reported. It is reported that the combination of 2 mg/L 2,4-D and 0.1 mg/L BAP gave the best callus induction for two rice varieties, Basmati-370 and Basmati-385 [19]. The same results were described for different

plants such as *Brassica napus* and *Piper betle* L. var Nigra [20,21]. Thus, it can be deduced that for a combination of 2,4-D and BAP, the growth of the callus was favored by a lower concentration of auxin and cytokinin, and the type of plant, cultivars as well as explants significantly influenced the concentration of auxin and cytokinin.

Table 5: Light condition for different cassava cultivars

Type of cultivars	Type of explants	Light condition	Reference
Rayong	Leaf Stem petiole	Continuous light, 16-h light, continuous dark	This study
Not available	Leaf Stem Root	16-h light (white cooling fluorescent lamps)	[8]
Doku Afisiafi Afebankye	Leaf Stem petiole Buds	Continuous dark	[12]
Soya Niargi Cololi Cacau Cacau roja	Not available	1st incubation: Continuous dark 2nd incubation in 13-h light (light intensity of 4000 lux)	[9]

Although the effect of the light is not significant to the callus growth based on the ANOVA results, it could still be observed that the cassava callus induced faster and had a higher frequency of callus formation under 24 hours of light exposure (started on the 12th day after inoculation) compared to the continuous dark condition (started on the 24th day). Callus formed under light condition have a frequency between 75% to 0% while the callus formed under total darkness gave a frequency between 50% to 0%. So far, studies related to the influence of light conditions on cassava callus culture are still scarce and different light conditions have been used to induce cassava callus culture as shown in Table 5. However, it is found that dark conditions are suitable for the initiation of rice callus culture while light exposure is suitable for its proliferation [22]. The results of this study partially conflicted with Chutipaijit [22] since the callus growth under 24 h light exposure recorded a better callus induction (75%) compared to the callus grown under continuous dark (50%), and 16h/8 light exposure (25%). On the other hand, a study by Ozarowski [23] on tobacco plants reported similar results to this study. The leaf blade of Motihari achieved 97.20% of callus formation under light condition. It was justified that the irregularities of reported results depend on the plant cultivar and type of explant used [23].

3.2 Preliminary Analysis of Starch Content in Callus

The amount of starch in cassava callus was determined using the cassava standard curve obtained from the quantitative iodine test performed on commercial cassava starch. The measured absorbance of the callus sample was 0.021 AU which corresponds to 0.22% of starch. This amount is relatively small compared to the standard amount (4.4 % starch) highlighted in Carciofi et al. [24]. The difference in value could be due to starch loss during the purification process. This statement is in line with our observation. Indeed, the small amount of callus obtained (0.0102 ±0.0002 g) went through several processes that need to be meticulously performed such as the separation of the supernatant and pallets during centrifugation. This

https://doi.org/10.31436/iiumej.v25i1.2585

process could cause some of the desired product to be washed off along with the supernatant if not carefully performed. Thus, the accuracy of the results is highly biased. For future work, the selection of the starch extraction process from cassava callus culture should be thoroughly studied.

Another reason that could cause the small amount of starch content in our cassava callus is due to the phase of callus growth (induction phase, proliferation phase or regeneration phase). A callus cycle comprises 3 phases: the induction phase (dedifferentiation and division of the explants of the cells), the proliferation phase (rapid division of the cells), and the regeneration phase (differentiation of the cells and organogenesis). It is reported that the callus accumulates sugar (glucose and starch) in the proliferation phase of the cycle for the regeneration phase as the callus will require large energy for organogenesis and to equilibrate the level of free soluble sugars to counterbalance the osmotic potential in the medium [25]. In this study, the callus was obtained from a 3-week-old subculture which means it was still in the early stage of proliferation explaining why the accumulation of the starch was lower as the callus did not accumulate enough starch for its regeneration phase.

Moreover, the callus obtained was smaller in size and yellow in color (Fig. 2). Those physical attributes indicate a low regenerable cultivar which is known to have small amounts of sucrose, glucose, and starch content [26]. For rice callus, it is stated that the TN1cultivars, which are non-regeneratable (NR), produce small and yellowish callus lacking glucose, sucrose, and starch content in both the callus induction phase and regeneration phase while the ANT39 cultivars, highly regeneratable (HR) cultivars, produce large, compact, and whitish callus which have high glucose, sucrose, and starch content in the callus induction phase but low sugar (glucose and starch) content in the regeneration phase. So far, no report regarding the regeneration of the cassava Rayong cultivars has been conducted. Thus, a conclusive statement cannot be provided since different plants have different attributes although they have the same capability to produce starch.

Fig. 2: Starch harvested from the callus induced from cassava stem explant in MS media with 8 mg/L of 2,4-D and 1 mg/L BAP.

4. CONCLUSION

It was found that the concentration of the PGRs as well as their combination highly affect the frequency of callus formation while the light condition has less to no significance on callus induction. It was also found that a lower concentration of 2,4-D and BAP are favorable to callus formation (8 mg/L 2,4-D: 1 mg/L BAP). Regarding the evaluation of the starch content in the cassava culture using an iodine test, it was found that the callus accumulates only 0.22 % of starch which is below the standard value of various callus (4.4%) and might be due to loss of starch during the purification process of the starch or the fact that the callus was in the early

stage of the callus cycle (early proliferation stage) or the regeneration power of the cultivar (NR cassava lack of starch content). Hence, more research is required to provide conclusive reasons for the lack of starch in the cassava callus culture. To improve the study of cassava callus initiation for starch production, it is highly recommended to select a sterilization technique that is suitable for all types of explants and to select a fast and effective extraction technique and starch analysis method for the evaluation of the amount of starch in cassava callus. It is hoped that this study will help in selecting the parameters for optimizing the culture conditions for cassava callus establishment towards starch production.

ACKNOWLEDGEMENT

We would like to acknowledge the Ministry of Higher Education Malaysia for funding this project under the Fundamental Research Grant Scheme (FRGS/1/2021/STG01/UIAM/02/2). In addition, the authors are grateful to the management of the International Islamic University Malaysia (IIUM) for a research fund (RMCG20-018-0018) for this work. Department of Chemical Engineering and Sustainability, Kulliyyah of Engineering, IIUM for providing the laboratory facilities to conduct this study. We also thanked NGP Technology Sdn. Bhd. for providing us with cassava cuttings to be planted at IIUM and then used as the explants to initiate cassava cultures.

REFERENCES

[1] Tan SL. (2015) Cassava – Silently, The Tuber Fills. UTAR Agriculture Science Journal, 1(2): 12-24.
[2] Li S, Cui Y, Zhou Y, Luo Z, Liu J, Zhao M. (2017) The industrial applications of cassava: current status, opportunities and prospects. Journal of the Science of Food and Agriculture, 97(8): 2282-2290. https://doi.org/10.1002/JSFA.8287
[3] Tumwesigye KS, Oliveira JC, Namuwaya S, Sousa-Gallagher MJ. (2021) Cassava Biomaterial Innovations for Industry Applications. In (Ed.), Cassava - Biology, Production, and Use. IntechOpen. https://doi.org/10.5772/intechopen.97493
[4] Cassava Starch Market, Consumption & Global Forecast. Available: https://www.renub.com/cassava-starch-market-p.php
[5] Howeler R, Cain P, Trumbore L, Hidajat SU. (2012) The challenge of large-scale cassava production. Sustainable Cassava Production in Asia for Multiple Uses and for Multiple Markets Proceedings 9th Regional Cassava Workshop, 313-321.
[6] NurulNahar E, Tan, SL. (2012) Cassava mini-cuttings as a source of planting material**,** J. Trop. Agric. and Fd. Sc., 40(1): 145-151.
[7] Hussain A, Ahmed I, Nazir H, Ullah I. (2012) Plant Tissue Culture: Current Status and Opportunities. Recent Advances in Plant in Vitro Culture, 37(3): 5-30. https://doi.org/10.5772/50568
[8] Abdalla N, Ragab M, El-Miniawy S, Taha H. (2013) Callus induction, regeneration and molecular characterization of cassava (*Manihot esculenta* Crantz). Journal of Applied Sciences Research, 9: 3781-3790.
[9] Faye A, Sagna M, Kane PD, Sane D. (2015) Effects of different hormones on organogenesis in vitro of some varieties of cassava (*Manihot esculenta* Crantz) grown in Senegal. African Journal of Plant Science, 9(8): 305-312. doi:https://doi.org/10.5897/AJPS2014.1243
[10] Mahdi H, Edward R. (2018) In vitro propagation of selected Malaysia cassava (*Manihot esculenta* Crantz) varieties by using nodal explants. Malaysian Applied Biology, 47(1): 1-5.
[11] Prammanee S. Kamprerasart K, Salakan S, Sriroth K. (2010) Growth and starch content evaluation on newly released cassava cultivars, Rayong 9, Rayong 7 and Rayong 80 at different harvest times. Kasetsart J., 44: 558-563.

[12] Fletcher EKA, Amoako TNE, Twumasi P. (2011) Effect of 2,4-D, explants type and cultivar on the callogenesis expression of cassava (*Manihot esculenta* Crantz) in Ghana. African Journal of Biotechnology, 10(46):9396-9401. https://doi.org/10.5897/ajb10.2115
[13] Puad NIM, Sofri NSNM, Amid A, Azmi AS. (2022) Optimization of Surface Sterilization Method and Initiation of Bitter Cassava Callus Culture. Biological and Natural Resources Engineering Journal, 6(1): 47-59.
[14] Puad NIM, Tang CW. (2015) A simple and Easy Method for Preparing Solid and Liquid Media for Plant Culture. Experimental Methods in Modern Biotechnology (vol. 3). IIUM Press, Malaysia.
[15] Reddy DK, Bhotmange MG. (2013) Isolation of Starch from Rice (Oryza sativa L.) and its Morphological Study using Scanning Electron Microscopy. International Journal of Agriculture and Food Science Technology, 4(9): 859-866.
[16] Bello OA, Esan EB, Obembe OO. (2018) Establishing surface sterilization protocol for nodal culture of Solanecio biafrae. IOP Conference Series: Earth and Environmental Science, 210: 012007. https://doi.org/10.1088/1755-1315/210/1/012007
[17] Hesami M, Naderi R, Tohidfar M, Yoosefzadeh-Najafabadi M. (2019) Modeling and Optimizing in vitro Sterilization of Chrysanthemum via Multilayer Perceptron-Non-dominated Sorting Genetic Algorithm-II (MLP-NSGAII). Frontiers in Plant Science, 10. https://doi.org/10.3389/fpls.2019.00282
[18] Anderson MJ, Whitcomb PJ. (2015) Two-level Factorial Design. DOE Simplified: Practical Tools for Effective Experimentation, www.statease.com.
[19] Ullah H, Ullah I, Jadoon SA, Rashid H. (2007) Tissue culture techniques for callus induction in rice. Sarhad J. Agric, 23(1), 81-86.
[20] Bano AS, Khattak AM, Basit A, Alam M, Shah ST, Ahmad N, Gilani SAQ, Ullah I, Anwar S, Mohamed HI. (2022) Callus Induction, Proliferation, Enhanced Secondary Metabolites Production and Antioxidants Activity of *Salvia moorcroftiana* L. as Influenced by Combinations of Auxin, Cytokinin and Melatonin. Brazilian Archives of Biology and Technology, 65: e22210200. https://doi.org/10.1590/1678-4324-2022210200
[21] Junairiah J, Purnomo P, Utami ESW, Ni'matuzahroh N, Sulistyorini, L. (2018) Callus Induction of Piper betle L. Var Nigra Using 2,4-Dichlorofenoxyacetic Acid and 6-Benzil Aminopurin. Biosaintifika: Journal of Biology & Biology Education, 10(3): 588-596. doi:https://doi.org/10.15294/biosaintifika.v10i3.15962
[22] Chutipaijit S, Sutjaritvorakul T. (2018) Titanium dioxide (TiO_2) nanoparticles induced callus induction and plant regeneration of indica rice cultivars (suphanburi1 and suphanburi90). Digest Journal of Nanomaterials and Biostructures, 13(4): 1003-1010.
[23] Ozarowski M. (2011) Influence of the physico-chemical factors, plant growth regulators, elicitors and type of explants on callus cultures of medicinal climbers of Passiflora l. L. Herba Polonica, 57(4): 58-75.
[24] Carciofi M, Blennow A, Nielsen MM, Holm PB, Hebelstrup KH. (2012) Barley callus: a model system for bioengineering of starch in cereals. Plant Methods, 8(1): 1-10. https://doi.org/10.1186/1746-4811-8-36
[25] Efferth T. (2019) Biotechnology Applications of Plant Callus Cultures. Engineering, 5(1): 50-59. https://doi.org/10.1016/j.eng.2018.11.006
[26] Lee ST, Huang WL. (2013) Cytokinin, auxin, and abscisic acid affects sucrose metabolism conduce to de novo shoot organogenesis in rice (*Oryza sativa* L.) callus. Botanical Studies, 54(1): 1-11. https://doi.org/10.1186/1999-3110-54-5