Jurnal
Teknologi

Full Paper

# THE EFFECT OF EXPLANT TYPE AND PICLORAM CONCENTRATION ON PRIMARY CALLUS INDUCTION AND SOMATIC EMBRYO FORMATION OF CASSAVA (*MANIHOT ESCULENTA* CRANTZ)

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**Article history**
Received
*14 July 2024*
Received in revised form
*26 September 2025*
Accepted
*14 October 2025*
Published Online
*16 June 2026*

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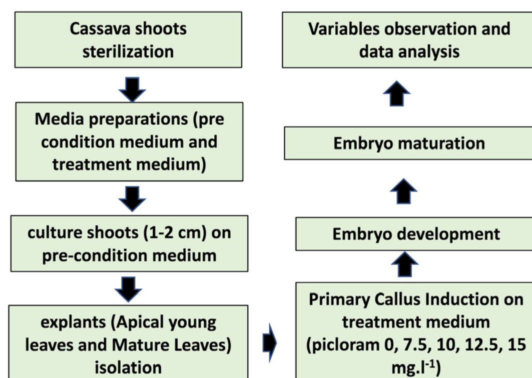
## Graphical abstract




## Abstract

Cassava (*Manihot esculenta* Crantz) is one of the leading agricultural commodities in Indonesia. Based on FAO records in 2016, cassava production reached up to 20,744,674 tons in Indonesia. Anticipating a demand of around 30 million tons of fresh cassava by 2025, an increase of cassava production by about 27% is necessary. One way to increase the productivity of cassava is by providing uniform and mass-produced cassava stem-cuttings from superior varieties. Somatic embryogenesis is a potential efficient method for cassava micropropagation. This research aimed to optimize the somatic embryogenesis induction and regeneration of cassava clone UJ 5. Two explant types, E1 (young leaf explants) and E2 (mature leaf explants), and four levels of picloram concentrations (M1=7.5 mg $l^{-1}$, M2=10 mg $l^{-1}$, M3=12.5 mg $l^{-1}$, and M4=15.0 mg $l^{-1}$ picloram) were tested. Each treatment supplemented with 6 mg $l^{-1}$ NAA. Results showed that the optimal explant for somatic embryo induction was identified from treatment utilizing E1 explants with M1 treatment. In contrast, the M4 treatment showed the optimal treatment for inducing somatic embryogenesis using E2 as explants. The somatic embryos developed E1 and E2 explants in all treatments showed similar subsequent developmental stages, i.e. globular, heart, torpedo, and cotyledon across both explant types, exhibiting varying percentages of embryo formation.

*Keywords*: Cassava, Somatic embryogenesis induction, embryo regeneration, Explant types, Picloram

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# 1.0 INTRODUCTION

Cassava, *Manihot esculenta* Crantz., is one of the most important carbohydrate-producing tuber crops for its wide utilization for food, starch-based industries, and bio-fuel [1]. Based on FAOSTAT 2024, Nigeria holds the top position globally in cassava production, followed by the Democratic Republic of Congo, Thailand, Ghana, Brazil, and Indonesia [2]. In 2017, Indonesia ranked third among cassava-producing countries, has since declined to sixth place in 2023 [2].

The challenges impacting cassava production, both in terms of quality and quantity, range from limited access to superior cassava varieties, issues related to propagule health and preparation, cultivation land degradation, environmental stress (biotic and abiotic), and technology limitation for production as well as postharvest management. In present, there are approximately 12 recognized cassava varieties, comprising sweet varieties (Adira 1, Malang 1, Malang 2, Darul Hidayah, and UK-1 Agritan), and bitter varieties (Adira 2, Adira 4, UJ 3, UJ 5, Malang 4, Malang 6, and Litbang UK-2) [3]. Cassava is mainly propagated by stem cuttings, which, due to their short-term storage capability, may inadvertently transfer pests and diseases to new plantation areas. Ensuring the availibility of sterile and healthy propagules in large quantities is critical to support sustainable cassava production.

Plant tissue culture is a laboratory-based method for growing plant cells, tissues, or organs in a sterile medium with the ultimate goal of regenerating them into whole plants suitable for field transfer [4]. This technique also facilitates plant breeding through various methods, including somaclonal variation, *in vitro* mutation, *in vitro* selection, protoplast fusion, and genetic transformation [5]. There are two main ways of plant cell regeneration in tissue culture: somatic embryogenesis and *de novo* organogenesis [6]. Somatic embryogenesis reveals the exchange in the somatic cell fate into an embryonic stem cell that usually be accomplished under hormonal induction (e.g., auxin), stress condition or modification of gene expression [6, 7].

In cassava, somatic embryogenesis is highly genotype-dependent and often exhibits low initiation of totipotent cell and conversion into plants [8]. Cassava totipotent cell initiation could be achived by optimizing embryo initiation medium (EIM), containing Murashige and Skoog basal medium [9], supplemented with picloram and 2,4-dichlorophenoxy acetic acid (2,4-D) to induce callus formation. After 21 days, the calli are transferred to an embryo maturation medium (EMM) for embryo maturation, and primary embryos are further developed into complete plants by either absisic acid pretreatment in EMM or by air dessication in the laminar air flow cabinet [10].

Cassava somatic embryogenesis is influenced by genotypes [10, 11,12] and the choice of explant type significantly affects the formation of friable embryogenic callus (FEC), with apical buds proving to be particularly effective [13]. Auxins play an important role in somatic embryogenesis [12]. Several reports revealed that picloram is an effective auxin for cassava somatic embryogenesis [8, 10, 11, 14]. However, the optimum picloram concentration should be determined for each genotypes to find the high rate of somatic embryo production and successful plant conversion.

Among the 12 cassava varieties cultivated in Indonesia, UJ-5 variety is recognized as a national superior variety, boasting high yields ranging from 25 to 38 tonnes $ha^{-1}$ [2]. UJ-5 serves as a reference variety [15] for productivity in cassava. Therefore, UJ-5 was chosen as the plant material for this study. Our research aimed to identify effective explant types and picloram concentrations in tissue culture medium supplemented with NAA for inducing somatic embryogenesis and regenerating UJ-5 cassava variety

# 2.0 METHODOLOGY

### Plant Materials and Explant Sterilization

Cassava UJ-5 cultivar which used as a source of explant was taken from Tanjung Bintang sub-district, South Lampung district, Lampung province, Indonesia. A stem cutting (25 - 30 cm in length) was planted in a polybag and incubated for 2 weeks in the greenhouse. The first to third sub apical nodes from axillary shoots were isolated. The explants were surface-sterilized with the protocol as follows: initially, around 5 cm of shoots were cleaned under running tap water for 60 minutes, followed by a 5-minutes soak in the detergent solution, then re-washed under running tap water, and transferred to a sterile bottle. Further sterilization was performed inside the laminar air flow cabinet (LAFC). In the LAFC, shoots which consisted of about 2-3 nodes were immersed in the solution of sodium hypochlorite (NaOCl 2 % v/v) for 15 minutes and rinsed 3 times with sterile water. The next sterilization steps were by soaking and gently shaking the shoots in a 70% (v/v) alcohol solution for a minute and continued rinsing the shoots with sterile water for 3 times. Eventually, the sterilized shoots were cut into approximately 1 cm lengths, with each cutting containing at least one node. These explants were cultured on pre-condition medium. Apical young leaves from shoots cultured for 2 weeks 3 x 5 $mm^2$ and mature leaves from shoots cultured for 6 weeks 5 x 5 $mm^2$ *in vitro* were subsequently used as explants for callus and somatic embryo induction.

### Medium Preparation

In this study, three types of media were used: pre-condition medium (PM), callus induction medium (CIM), and somatic embryogenesis maturation medium (SEMM). Murashige and Skoog (MS) basal salt medium (PhytoTech Lab Company, United States) was

used for all media formulations. PM was used initially to culture one node axillary shoot following shoot sterilization. PM contained MS basal medium without any plant growth regulators (PGRs). The CIM formulation included MS basal salt medium supplemented with NAA 6 mg $l^{-1}$ and various concentrations of picloram: 7.5, 10.0, 12.5, and 15.0 mg $l^{-1}$. The SEMM medium shared similar PGRs as CIM but at reduced concentrations of 6 mg $l^{-1}$ picloram and 0.5 mg $l^{-1}$ NAA. Each medium was added with sucrose 30 g $l^{-1}$ for PM, 40 g $l^{-1}$ for both CIM and SEMM. In addition, $CuSO_4$ 4 µM was included in CIM and SEMM. Each medium pH was adjusted to 5.8.

### Culture Procedure

#### *Primary Callus Induction*

Leaf explants (apical young leaves and mature leaves) were obtained from sterile shoots grown *in vitro*. Apical young and mature leaves were excised 5 x 5 $mm^2$ segments and cultured on CIM medium. Each bottle contained 3 explants and was incubated in darkness at 25 ± 2°C for six weeks, with subculturing performed at the fourth week using the same medium composition. Observations of callus were done at 4 weeks after induction (WAI), including the measurement of callus fresh weight, percentage of primary callus induction [12], and visual observation of embryogenic callus formation under an Olympus binocular microscope (Olympus, Tokyo, Japan).

#### *Embryo Development and Maturation*

The callus grown on CIM medium was subsequently divided into approximately 0.2 g portions and sub-cultured into SEMM medium, with each bottle containing three calli. These calli was incubated in darkness at 25 ± 2°C for a minimum three weeks. After four weeks development, the percentage of callus formation (number of explants forming primary callus) was assessed. On the other hand, the embryo developments were visually inspected under a microscope (Olympus, Tokyo, Japan) to identify stages of embryos structure (globular, heart, torpedo and cotyledon stage). Embryos that did not reach the cotyledonary stage were maintained in SEMM under the same incubation conditions as the previous callus culture.

### Research Design

A completely randomized design (CRD) arranged in a factorial (2 x 4) format was employed in this study. The first factor was explants type (E), consisting of two levels E1 = apical young leaves and E2 = mature leaves. The second factor was the concentration of picloram (M) which consisted of 4 levels: M1 = 7.5 mg $l^{-1}$ picloram + 6 mg $l^{-1}$ NAA, M2 = 10.0 mg $l^{-1}$ picloram + 6 mg $l^{-1}$ NAA, M3 = 12.5 mg $l^{-1}$ picloram + 6 mg $l^{-1}$ NAA, and M4 = 15.0 mg $l^{-1}$ picloram + 6 mg $l^{-1}$ NAA. Each treatment was replicated 3 times, with each replication comprising 3 bottles and each bottle containing three explants. Therefore, a total of 216 explants were used in this study.

### Data Collection and Analysis

The callus fresh weight was measured at 4 WAI by weighing of callus at least 6 calli for each treatment. Callus percentage refers to the number of callus formations relative to the total number of explants cultured. Meanwhile, observations on embryo formation included the embryo percentage, which denotes the number of embryo formation developed from callus on SEMM medium. Visualizations were conducted to monitor explant growth and the formation of each embryo stage. The collected data were subjected to Analysis of Variance (ANOVA). If the treatments exhibited significant differences, post-hoc Least Significant Difference (LSD) test were performed at the 5% significant level to determine specific differences among treatments.

## 3.0 RESULTS AND DISCUSSION

### Primary Callus Visual Observation

Callus growth was monitored every 2 days following incubation of explants on callus induction medium (CIM). Visual observations revealed distinct differences in callus formation between young leaf explants (E1) and mature leaf explants (E2), particularly in terms of initial appearances, callus size, and callus formation rate during the first week. Callus formation on E1 began visibly by the 7th day post induction, characterized by the enlargement of explant size. This enlargement indicated cellular growth and proliferation in response to the culture medium. Furthermore, by one week after induction (WAI), noticeable swelling of excised leaves was observed on both sides, indicating initiation of callus growth. In contrast, E2 showed only an increase in explant size by the 4th day, with no visible callus formation observed until the first week across all concentrations of primary callus induction medium (Figure 1). In addition, E1 underwent a color transition from green to yellowish-green, while E2 remined green. E2 initiated callus formation later than E1, approximately 14 days after induction (DAI), which was one week behind E1. Explant is a piece of meristematic tissue or an organ that is extracted from the living organism serves as a starting planting material for in vitro culture. Young leaves explant such as leaf lobes or axillary buds has been reported effectively to induce callus on several African cassava cultivars [16]. In this study, both explant used were placed in abaxial position on the medium. Placing the explant in abaxial or adaxial position, callus was beginning occurred at the cutting site region and proliferated to the entire explant and started appearing globular structure of embryo after frequently subculture. On the other hand, at the larger

explant size, embryogenic callus was unable to produce, instead they formed hard watery callus or callus structure like white watery cotton [17].

The performance of callus derived from both explant types at 4 WAI is illustrated in Figure 2. Both E1 and E2 exhibited vigorous callus growth, such that by the 4th week, observable differences in callus appearances between E1 and E2 had disappeared. The presence of callus in both explant types across all picloram concentrations in the primary callus induction medium indicated the embryogenic callus.

### Visualization of Somatic Embryos

Embryogenic callus, defined by its potential to develop into somatic embryos, is characterized by a friable yellowish crumb structure. After 4 weeks on callus induction medium (CIM), the primary callus was excised at approximately 0.2 g in size and subsequently sub-cultured to somatic embryogenesis maturation medium (SEMM). The observation revealed that some explants have produced globular stage of embryos after 3-4 weeks in callus induction media. Subsequently, embryos develop into the heart, torpedo, and cotyledon phases after being transfer to somatic embryogenesis maturation medium within 2 weeks with asynchronous structures (Figure 3), which is possibly caused by an imbalance nutrients uptake from the media into plant tissues [18].

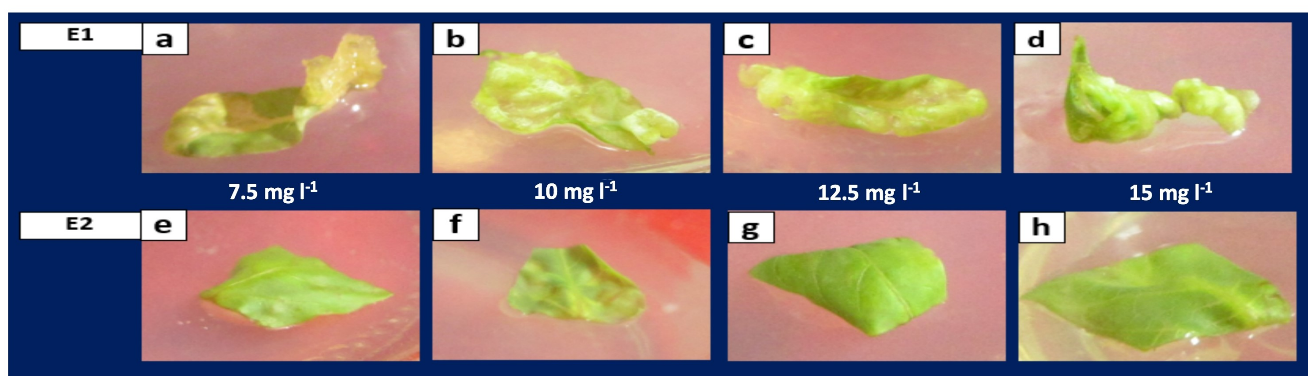


**Figure 1** The visual appearance of UJ-5 cassava leaf explants in MS basal medium with various picloram concentrations (7.5, 10, 12.5, and 15 mg $l^{-1}$) supplemented with NAA 6 mg $l^{-1}$at one week after induction (WAI) (E1: young leaf explants, E2: mature leaf explants)

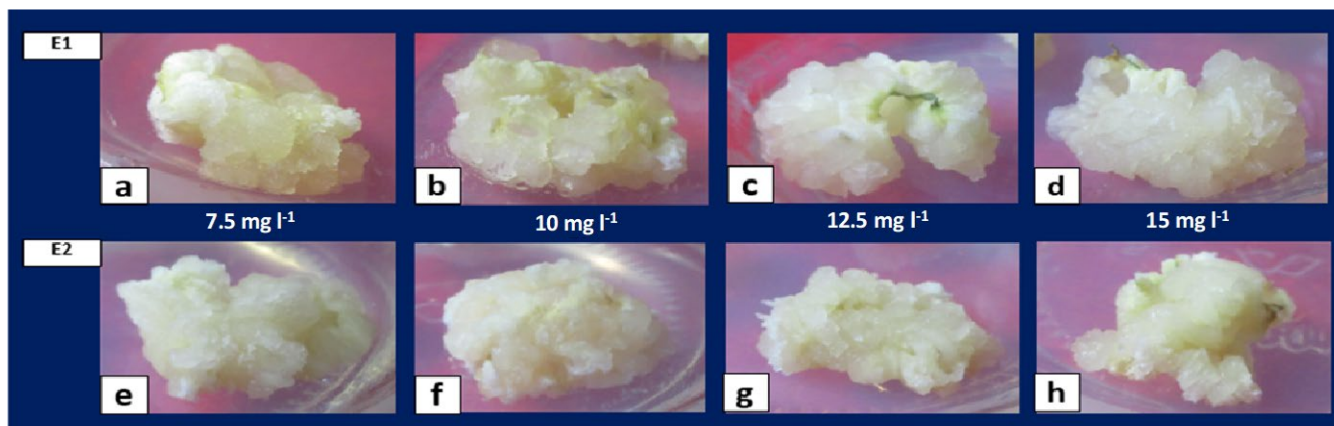


**Figure 2** The visual appearance of callus developed from different explant types in MS basal medium with various picloram concentrations (7.5, 10, 12.5, and 15 mg $l^{-1}$) at 4 weeks after induction (WAI). E1 = young leaf explants; E2 = mature leaf explants.

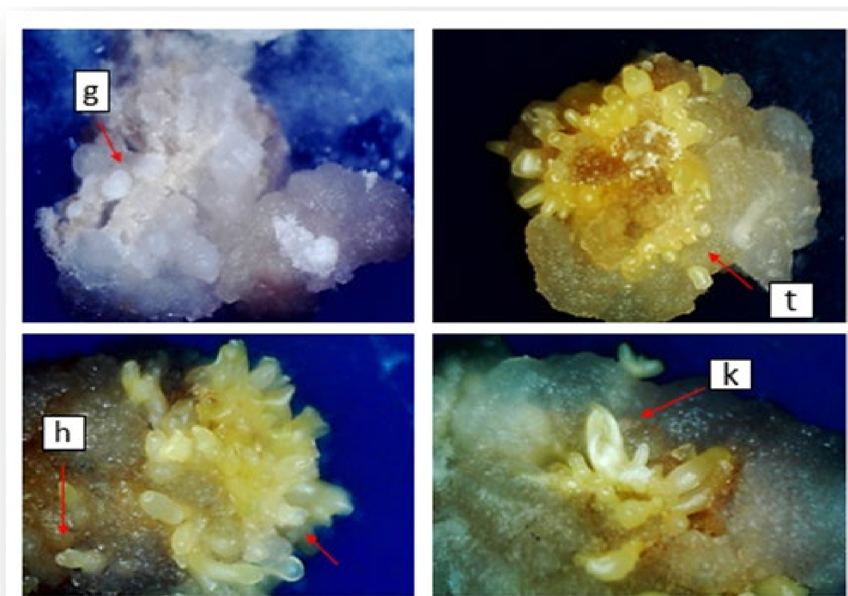


**Figure 3** Developmental stages of embryos developed from young leaf explants (E1): (g) globular stage, (h) heart stage, (t) torpedo stage, (k) cotyledon stage

## Percentage of Callus Formation (4 WAI)

Two explants type used in this study were equally effective on callus induction. The analysis of variance revealed that no significant different caused by type of explant, picloram concentration and the interaction between them on the number of explants forming callus within 4 weeks of induction. All types of explants were able to produce callus reached 100% at all concentrations of picloram tested (Table 1). In this study, the percentage of callus induction was higher than earlier report on cassava UJ3 and BW1 [12] as well as Adira 4, Malang 6 and Sutera [19]. The variations in callus formation and growth among varieties might result from genetic difference.

## Primary Callus Fresh Weight at 4 WAI

The analysis of variance (ANOVA) on the fresh weight of callus at 4 weeks after induction (WAI) indicated significant differences due to explant types, picloram concentrations, and their interaction. According to the Least Significant Difference (LSD) test at the 5% level, the highest fresh weight of primary callus (1.88 g) was observed in young leaf explants (E1) at a picloram concentration of 7.5 mg $l^{-1}$. In contrast, mature leaf explants (E2) produced a callus fresh weight of 1.73 g at a picloram concentration of 15 mg $l^{-1}$. These results suggest that the optimal picloram concentration for inducing primary callus in young leaf explants is 7.5 mg $l^{-1}$, whereas mature leaf explants respond better to a concentration of 15 mg $l^{-1}$ (Table 2). Our result showed that each genotype has a different response to PGRs concentration applied. In the previous report, [20] observed that heavier callus produced by certain genotype in peach plant is due to the greater sensitivity of tissue to plant growth regulator and explant selection plays important role in producing callus as explant respond to the medium is highly depend on genotype and physiological stage. Therefore, different types of explants for any given species do not respond equally, resulting in different induction levels of embryogenic callus [21]

**Table 1** The effect of explant types and picloram concentrations on callus percentage at 4 weeks after induction (WAI)

| Picloram concentrations (mg $l^{-1}$) | Explant types | Callus percentage (%) |
|---|---|---|
| 7.5 | E1<br>E2 | 100<br>100 |
| 10 | E1<br>E2 | 100<br>100 |
| 12.5 | E1<br>E2 | 100<br>100 |
| 15 | E1<br>E2 | 100<br>100 |

**Table 2** The effect of Interaction of explant types and picloram concentrations on fresh weight of primary callus at 4 weeks after induction (WAI)

| Picloram concentrations (mg l⁻¹) | Explant types | |
|---|---|---|
| | E1 | E2 |
| 7.5 | 1.88 ± 0.11 a A | 0.66 ± 0.12 b B |
| 10 | 1.52 ± 0.13 a B | 0.45 ± 0.17 b B |
| 12.5 | 1.08 ± 0.01 a C | 0.59 ± 0.04 b B |
| 15 | 1.15 ± 0.16 b C | 1.73 ± 0.24 a A |

Numbers followed by the same letter are not significantly different according to the 5% LSD test. Lowercase letters indicate horizontal comparisons and uppercase letters indicate vertical comparisons. Value of LSD 5% = 0.25. E1 = young leaf explants; E2 = mature leaf explants.

**Table 3** The effect of explant types and picloram concentrations on the percentages of developed embryos

| Picloram concentrations (mg l⁻¹) | Embryo Percentage (%) | |
|---|---|---|
| | E1 | E2 |
| 7.5 | 25 | 0 |
| 10 | 16.7 | 8.3 |
| 12.5 | 16.7 | 0 |
| 15 | 8.3 | 8.3 |

E1= young leaf explants; E2= mature leaf explants

## Embryo Percentages

The percentage of embryos was calculated based on the number of explants produced embryos. Young leaf explants (E1) yielded the highest percentage of embryos (25%) in MS medium containing picloram 7.5 mg l⁻¹. Increasing the concentration of picloram led to a decrease in the percentage of embryos. On the contrary, mature leaf explants showed the highest percentage of embryos (8.3%) at picloram concentrations of 10 mg l⁻¹ and 15 mg l⁻¹ (Table 3).

Visual observation of the callus revealed callus morphological changes during induction. Callus induction began with the swelling of the explants and an increase the size of leaf explants due to the medium imbibition. Swelling on the explants was caused by the absorption of growth regulators and nutrients from the medium into the actively dividing (meristematic) tissues in young leaf explants [22]. This swelling was followed by the appearance of callus on the explants. In addition, factors influencing cell division in explants include the sucrose content in the medium. Sucrose acts as an energy source for accelerating cell division. Additionally, sucrose plays an important role in the diffusion of nutrients from the medium to the explants by maintaining osmotic pressure, as the concentration gradient of the medium is higher than that of the explants [22].

Embryogenesis begins with the formation of a primary callus, characterized by swelling in the leaf veins on the leaf surface and at the cut end of the explant. Callus induction begins with the thickening of the explants (cassava leaves) at the cut and wound-affected areas. This thickening results from the interaction between explants, growth media, growth regulators, and environment conditions [23]. The appearance of callus on explants is marked by the appearance of clear white tissue at the incisions and on the surface of the explants, which then develops into small, clear white spheres and eventually forming callus.

At 4 weeks after induction, the callus formed in the *in vitro* culture of the UJ-5 was clear-white, watery, and crumbly. This observation is in accordance with the research [23], who reported that callus from young leaf explants and shoots exhibits a crumbly structure, while callus from petiolate explants has a compact and rigid structure. Based on texture and cell composition, callus can be categorized into compact and crumbly types. Compact callus has a dense and hard texture, which is composed of very small cells tightly packed together. In contrast, crumbly callus has a soft texture and are composed of cells with a lot of intercellular space [24]. In this study, the callus structure formed from the UJ-5 clone was identified as crumbly callus. Crumbly callus does not undergo lignification, whereas compact callus lignifies due to

the influence of growth regulators, which make callus become harder [25]. Therefore, crumbly callus is generally more suitable for culture in suspension media compared to compact callus [13].

The activity of cell division in the callus can be seen from the color of the callus, which reflects the visual appearance of callus cells. Observation results showed that all callus initially formed was white. The color of the callus remained white up to the 4th week, after which it gradually changed to yellowish-white and then to brown. This color change in the callus indicates a transition in cell growth phases, from young and actively dividing cells (white) to mature cells (yellowish white) [13].

## 4.0 CONCLUSION

In the present study, the protocol for somatic embryogenesis of the cassava UJ-5 variety was developed. Young leaf explants were found to be more responsive in inducing embryogenic callus compared to mature leaf explants. Picloram at a concentration of 7.5 mg $l^{-1}$ was the most effective plant growth regulator for inducing primary callus fresh weight in young leaf explants. In contrast, for mature leaf explants, the optimal concentration for callus weight induction was 15 mg $l^{-1}$ picloram. Additionally, young leaf explants produced a higher percentage of embryos compared to mature leaf explants.

## Acknowledgement

The authors would like to thank the Department of Agrotechnology, Faculty of Agriculture, University of Lampung, for providing the opportunity to conduct research. Also, the authors would like to thank students and all cassava research team at the Faculty of Agriculture University of Lampung and the Bogor Agricultural University through the National Collaborative Research Scheme of the Higher Education Endowment Fund Program Towards World University Rankings with the title: Identification of Genotypes of Cassava Resistant to Low pH Stress Using Nutrient Culture Techniques to Support the Development of Cassava Varieties in Marginal Land, according to Contract Number: 482/IT3. D10/PT.01.03/P/B/2023 with Chief Researcher Prof. Dr. Dewi Sukma, S.P., M.Si.

## Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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