

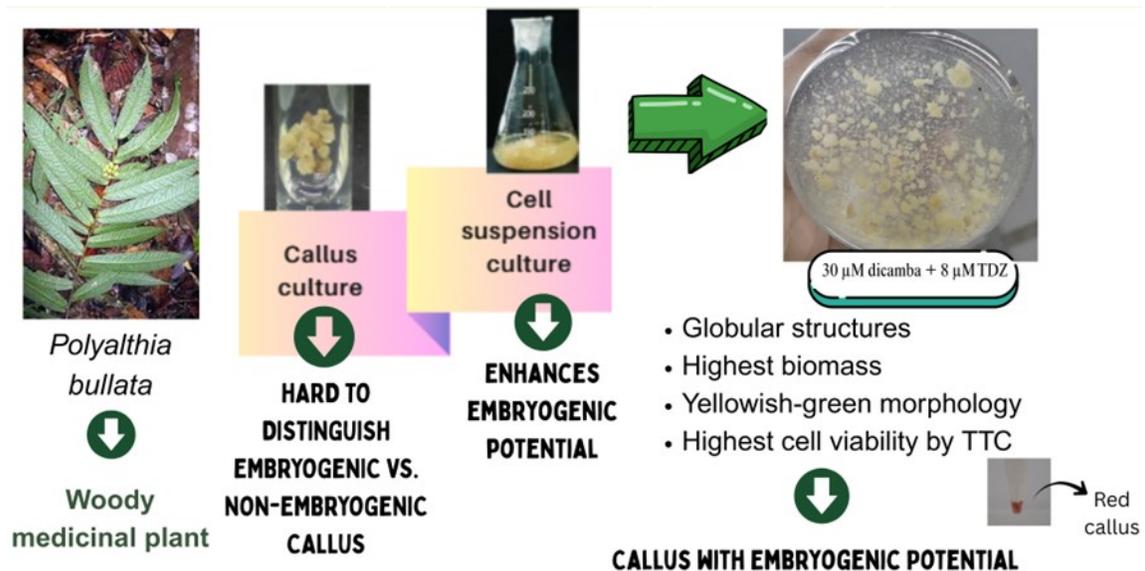
# Colorimetric Evaluation of Callus with Embryogenic Potential and Biomass Accumulation in the Woody Medicinal Plant *Polyalthia bullata* Suspension Culture

Nancy N. Marianyagam , Munirah Adibah K. Zaman , Noor A. Shahrudin , and Azzreena M. Azzeme ,\*

\*Corresponding author: azzreena@upm.edu.my

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## GRAPHICAL ABSTRACT



# Colorimetric Evaluation of Callus with Embryogenic Potential and Biomass Accumulation in the Woody Medicinal Plant *Polyalthia bullata* Suspension Culture

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Identifying embryogenic callus in plant tissue culture, particularly in woody species, such as *Polyalthia bullata*, is often hindered by its morphological similarity to non-embryogenic callus. This study introduced a rapid, cost-effective colorimetric method using the triphenyl tetrazolium chloride (TTC) assay to evaluate embryogenic callus induction in *P. bullata* via cell suspension culture. Suspension cultures were established using 30  $\mu$ M dicamba in combination with varying concentrations of thidiazuron (TDZ: 2 to 10  $\mu$ M). Among the treatments, 30  $\mu$ M dicamba with 8  $\mu$ M TDZ induced the highest biomass and a distinct yellowish green callus morphology, typically associated with embryogenic potential. The TTC assay confirmed high cell viability, with red staining indicating elevated metabolic activity in embryogenic cultures. These findings validate the TTC assay as an efficient proxy for embryogenic callus detection, providing a valuable alternative to labor-intensive histological or molecular methods. This approach enhances tissue culture protocols for this woody medicinal plant, supporting its large-scale propagation, biomass productivity, and conservation.

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Keywords: Auxin; Biomass; Cytokinin; Embryogenic cell suspension; *Polyalthia bullata*

Contact information: Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia; \*Corresponding author: azzreena@upm.edu.my

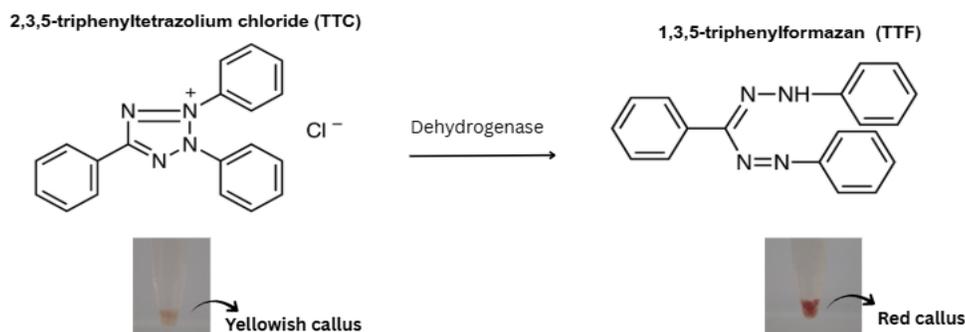
## INTRODUCTION

*Polyalthia bullata* is a woody medicinal plant of high therapeutic value. It is traditionally used for its antimicrobial, antioxidant, and anticancer properties. Its bioactive compounds have shown promise in treating various ailments, generating interest in its large-scale propagation for pharmaceutical development and conservation purposes (Kamarul Zaman *et al.* 2020a, 2020b, 2021). However, tissue culture-based propagation of *P. bullata* remains challenging, particularly due to difficulties in distinguishing embryogenic callus from non-embryogenic callus, as both often exhibit similar morphological characteristics. This limitation compromises the accuracy of callus selection, thereby reducing regeneration efficiency and hindering the development of optimized protocols. Moreover, cell suspension culture was employed *in lieu* of callus culture, as it yields a uniform population of rapidly proliferating cells that exhibit enhanced interaction with nutrients and growth regulators, hence augmenting embryogenic potential and facilitating large-scale propagation (Kong *et al.* 2020).

Plant growth regulators, especially auxins and cytokinins, play a critical role in inducing embryogenic callus in many plant species. Dicamba, a synthetic auxin, has been reported to induce embryogenic callus in *Triticum aestivum* L. (Ren *et al.* 2010) and *Zea mays* L. (Akoyi *et al.* 2013). Thidiazuron (TDZ), a phenylurea compound with cytokinin-like activity, has proven effective in promoting somatic embryogenesis in *Vaccinium corymbosum* × *V. angustifolium* (Ghosh *et al.* 2018), *Simmondsia chinensis* (El-Ashry *et al.* 2017), and *Saccharum* spp. (Gallo-Meagher *et al.* 2000). While hormonal strategies have improved embryogenesis, the identification of callus with embryogenic potential still depends on conventional methods such as histological observation or molecular analysis.

Although precise, these traditional techniques are labor-intensive, time-consuming, and often require costly reagents and skilled personnel. Histological analysis involves complex sample preparation steps including fixation, embedding, sectioning, and staining, while molecular methods, such as polymerase chain reaction (PCR) or gene expression profiling demand advanced laboratory infrastructure (Mikuła *et al.* 2010; Kaushal and Sidana 2018). Such limitations reduce their practicality for large-scale or routine applications in tissue culture laboratories.

To address this bottleneck, the present study explores a TTC-based colorimetric assay as a rapid and practical alternative for identifying embryogenic callus in *P. bullata* suspension cultures. It is hypothesized that the TTC (2,3,5-triphenyltetrazolium chloride) is reduced by dehydrogenase enzymes in viable cells to produce red formazan, serving as a reliable visual marker of metabolic activity and cell viability, as shown in Fig. 1 (Wang *et al.* 2023). When paired with morphological indicators, such as yellowish-green coloration, commonly associated with embryogenic competence, TTC offers a simple, non-destructive screening tool for the identification of embryogenic callus characterized by intense red staining which indicates a higher glycolytic activity and metabolic rate in viable enzymatic tissues.



**Fig. 1.** Schematic representation of the TTC reduction reaction. Viable *P. bullata* callus reduces colorless TTC to red formazan (TTF) through dehydrogenase enzyme activity

This study is the first to develop a TTC-based colorimetric method tailored to *P. bullata*. Through optimizing auxin–cytokinin combinations and validating results through visual morphology, viability staining, and microscopic examination, this work presents a novel and scalable strategy for enhancing tissue culture protocols for recalcitrant woody medicinal plants.

## EXPERIMENTAL

### Plant Materials

The research was performed in the Plant Biochemistry and Biotechnology Laboratory at the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, Serdang, Selangor. The calli were derived from the *P. bullata* leaf with the identification number of PID 170820-13, provided by the Forest Research Institute Malaysia (FRIM).

### Multiplication of *P. bullata* Callus in MS Media Supplemented with 30 $\mu$ M Dicamba

The callus was multiplied using MS media (Murashige and Skoog 1962) containing 30  $\mu$ M dicamba. All equipment used were autoclaved before subculturing callus on MS media supplemented with dicamba under laminar air-flow cabinet. Flasks containing callus were wrapped with aluminum foil and then sealed with plastic wrap to be incubated under dark condition for 3 weeks at  $24 \pm 2$  °C.

### Multiplication of *P. bullata* Cell Suspension Culture

Based on preliminary study (data not shown), a series of treatments were tested to determine the optimal combination of plant growth regulators for inducing embryogenic cell suspension culture in *P. bullata*. The experiment involved using two auxins, 30  $\mu$ M dicamba and 30  $\mu$ M NAA, in combination with three cytokinins at concentrations of 2  $\mu$ M TDZ, 2  $\mu$ M BAP, and 2  $\mu$ M kinetin. The effects of these combinations were evaluated based on the increase in fresh and dry weight of the cultures and morphology of suspension cultures. Among the treatments, the combination of 30  $\mu$ M dicamba and 2  $\mu$ M TDZ exhibited the highest increase in both fresh and dry weights as well as favourable morphology, indicating high embryogenic potential. Based on these findings, the final study focused on optimizing the concentrations of TDZ while maintaining dicamba at a fixed concentration of 30  $\mu$ M to further enhance the induction and development of embryogenic cell suspension culture.

One gram of 3-week-old friable calli was cultured in 100-mL conical flask containing 25 mL of  $\frac{1}{2}$  MS media supplemented with hormonal combinations as described in Table 1. The flasks were wrapped with aluminum foil and placed on orbital shaker at 120 rpm at  $24 \pm 2$  °C for 18 days (Farezol *et al.* 2024). Based on a study by Pinto (2023), the growth of *P. bullata* cells in cell suspension culture had entered stationary phase after incubation for 18 days.

### Determination of Cell Suspension Growth

The growth of cell suspension was determined by constructing a growth curve based on the data from the measurement of fresh weight and dry weight of suspension cells (Pinto 2023). The cell suspension was harvested by filtering the cells with filter paper (Whatman No. 1). The initial weight of a clean and dry container was initially recorded. By scraping the cells with a spatula, the filtered cells were transferred to the pre-weighed container to measure the fresh weight. For determination of dry weight, the cells were dried in an oven at 50 °C for 2 days or until the constant weight was reached to determine the dry weight. The calculation for the dry weight of cells was done using Eq. 1:

$$\text{Dry weight (g)} = \text{Final weight with cells (g)} - \text{Weight of container (g)} \quad (1)$$

The growth curve was constructed based on the data recorded for both fresh and dry weight. Moreover, the data were recorded at 3-day intervals for 18 days.

## Identification of Embryogenic Callus

### *Microscopic analysis*

The embryogenic callus formations were observed using a light microscope (Zeiss Stemi DV4; Carl Zeiss Microscopy, Göttingen, Germany). The calli in the suspension cultures were removed from the flasks and then blotted dry on a sterile tissue paper. Afterwards, the embryogenic tissue portions were positioned on a glass slide. The appearance of the cells as well as the arrangement and organization of the cells were observed under a microscope. The callus with embryogenic potential is identified by the presence of isodiametric clusters of cells; these clumps are spherical globules surrounded by a layer of organised cells (Woo *et al.* 2021).

## Determination of Cell Viability

### *Morphology of cell suspension culture*

The morphology of cell suspension cultures of all treatments throughout the 18 days period of incubation were observed and recorded at 3-day intervals.

### *TTC test*

To assess the viability of the cells in suspension cultures, a TTC assay was carried out (Nagananda and Satishchandra 2023). The TTC solution of 1% was prepared by dissolving 0.2 g in 20 mL sterile phosphate buffer. Then, the pH of the solution was adjusted to 7.5 using potassium hydroxide (KOH). Afterwards, TTC solution was stored in the dark at 4 °C. A total of 500 µL of cultures were transferred into microcentrifuge tubes. After addition of 1 mL of TTC to the tubes, the tubes were incubated at room temperature in dark conditions. After 1 h, the colour change was observed and recorded.

## RESULTS AND DISCUSSION

### Establishment of Cell Suspension Culture

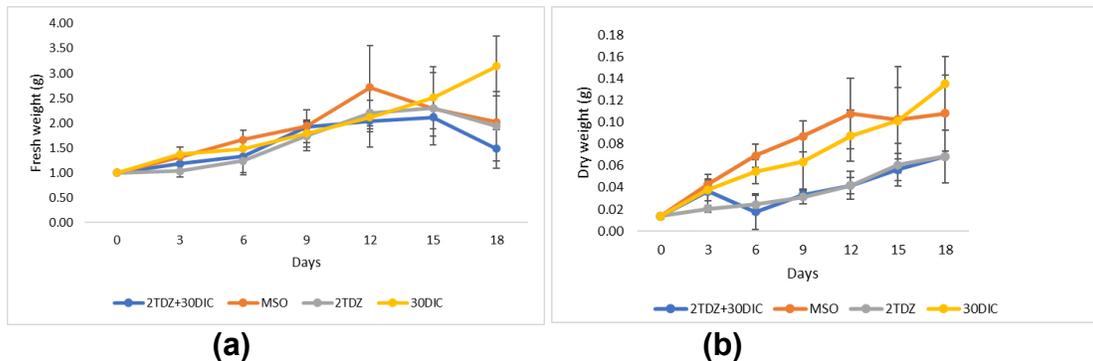
The effects of varying concentrations of dicamba and TDZ on biomass accumulation in *P. bullata* suspension cultures were assessed over an 18-day period (Figs. 1 and 2). Cultures maintained in MS medium without hormones (MSO) showed limited proliferation, with fresh weight peaking at day 12 ( $2.715 \pm 0.839$  g) before declining, reflecting the absence of exogenous hormonal stimulation.

In treatments where auxin was the only hormone, 30 µM dicamba resulted in the highest biomass accumulation by day 18 ( $3.144 \pm 0.603$  g FW and  $0.135 \pm 0.025$  g DW), consistent with its role in promoting cell division and callus proliferation (Dong *et al.* 2024). However, this likely favored non-embryogenic callus formation due to the absence of cytokinin.

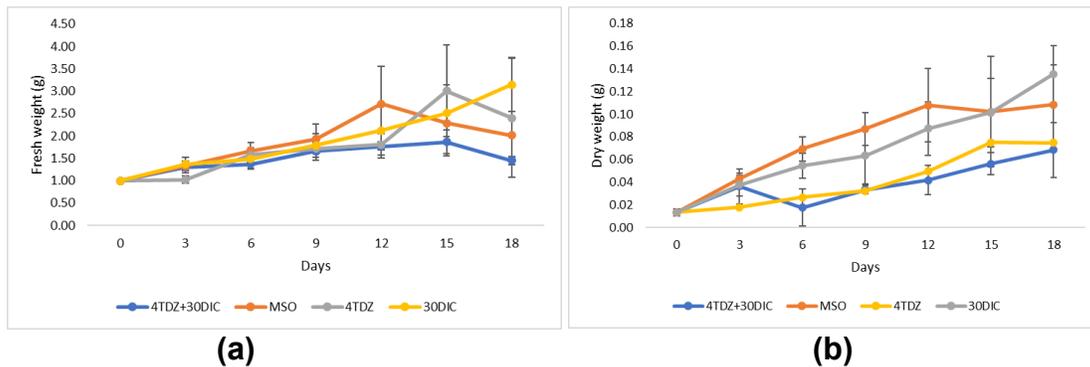
When applied alone, TDZ (2 to 10 µM) resulted in variable growth. Notably, 4 µM TDZ yielded the highest fresh weight ( $3.009 \pm 1.017$  g FW) on day 15, while 10 µM TDZ produced steady increase in dry biomass, suggesting its role in morphogenesis and biomass stability (Usman *et al.* 2022; Zaytseva *et al.* 2022). However, higher TDZ concentrations

also slowed initial growth, possibly due to inhibitory effects on early cell proliferation (Bernula *et al.* 2020).

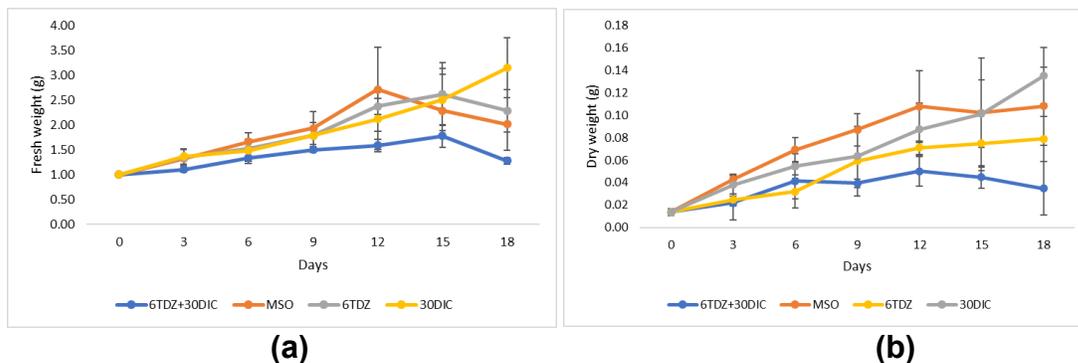
Auxin-cytokinin combinations demonstrated synergistic effects. The combination of 30  $\mu\text{M}$  dicamba with 10  $\mu\text{M}$  TDZ produced the highest fresh weight ( $2.258 \pm 0.363$  g FW), whereas the 8  $\mu\text{M}$  TDZ combination yielded the highest dry weight ( $0.087 \pm 0.025$  g DW) on day 18. These results support the importance of balanced auxin-cytokinin ratios in promoting embryogenic biomass accumulation (Tripathi *et al.* 2021; Kaňuková *et al.* 2024).



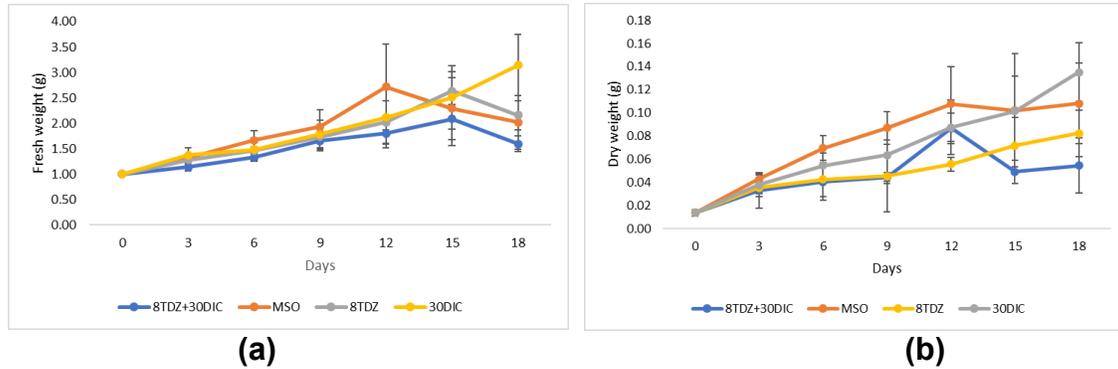
**Fig. 2.** Growth curve of fresh weight (a) and dry weight (b) in *P. bullata* cell suspensions cultured in  $\frac{1}{2}$  MS medium supplemented with 2  $\mu\text{M}$  TDZ and 30  $\mu\text{M}$  Dicamba over 18 days



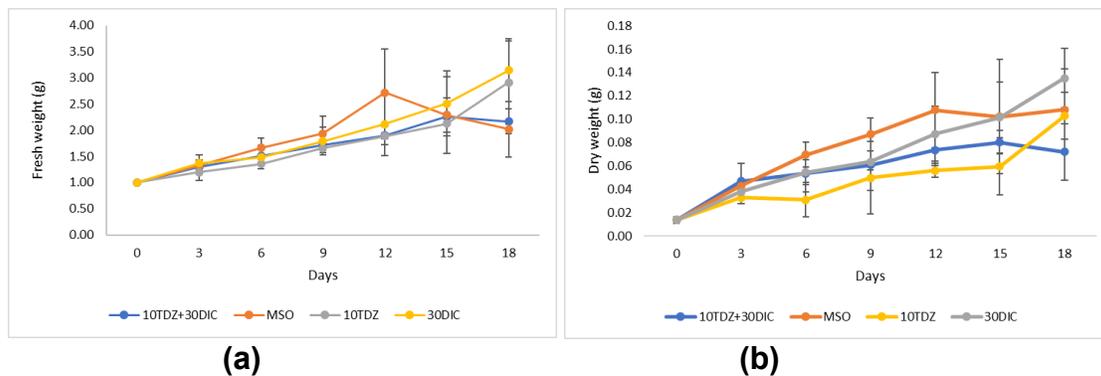
**Fig. 3.** Growth curve of fresh weight (a) and dry weight (b) in *P. bullata* cell suspensions cultured in  $\frac{1}{2}$  MS medium supplemented with 4  $\mu\text{M}$  TDZ and 30  $\mu\text{M}$  Dicamba over 18 days



**Fig. 4.** Growth curve of fresh weight (a) and dry weight (b) in *P. bullata* cell suspensions cultured in  $\frac{1}{2}$  MS medium supplemented with 6  $\mu\text{M}$  TDZ and 30  $\mu\text{M}$  Dicamba over 18 days



**Fig. 5.** Growth curve of fresh weight (a) and dry weight (b) in *P. bullata* cell suspensions cultured in  $\frac{1}{2}$  MS medium supplemented with 8  $\mu$ M TDZ and 30  $\mu$ M Dicamba over 18 days



**Fig. 6.** Growth curve of fresh weight (a) and dry weight (b) in *P. bullata* cell suspensions cultured in  $\frac{1}{2}$  MS medium supplemented with 10  $\mu$ M TDZ and 30  $\mu$ M Dicamba over 18 days

Growth patterns were consistent with typical suspension culture dynamics, exhibiting lag (0 to 3 days), exponential (3 to 12 days), stationary (12 to 15 days), and aging (15 to 18 days).

This pattern is consistent with findings reported in *Pinus thunbergii* and *P. europaea* suspension cultures (Beigmohammadi *et al.* 2019; Sun *et al.* 2024). Notably, the extended exponential phase observed in *P. bullata* suggests a high regenerative capacity under optimal hormonal conditions.

### Morphological Indicators of Embryogenic Potential

Changes in culture color served as visual cues of cellular differentiation and viability (Table 1). The MSO and dicamba-only cultures gradually turned yellowish-brown, consistent with stress-induced phenolic accumulation common in woody species (Gemechu and Amante 2021; Permadi *et al.* 2024). Similarly, TDZ-only treatments followed the same browning pattern, suggesting that cytokinin alone was insufficient to sustain embryogenic development.

In contrast, the 30  $\mu$ M dicamba + 8  $\mu$ M TDZ treatment retained a yellowish-green color from day 9 to 15, which is a visual trait often associated with embryogenic potential (Mishra *et al.* 2021; Hamza *et al.* 2024). This observation was further substantiated by TTC viability staining and microscopy.

### Validation by TTC Assay and Microscopy

The TTC assay provided a rapid colorimetric method to evaluate metabolic activity and embryogenic viability. Cultures treated with 30  $\mu\text{M}$  dicamba + 8  $\mu\text{M}$  TDZ produced the most intense red staining by day 12 (Table 2), reflecting high dehydrogenase activity in viable, embryogenic cells (Wang *et al.* 2023). Lower intensity in 2 and 4  $\mu\text{M}$  TDZ combinations indicated moderate activity, while 30  $\mu\text{M}$  dicamba alone and 10  $\mu\text{M}$  TDZ treatments showed diminished staining, potentially due to stress or cytotoxicity (Naseem *et al.* 2020).

Microscopic analysis further confirmed these findings, revealing globular structures characteristic of early-stage somatic embryo in the 30  $\mu\text{M}$  dicamba + 8  $\mu\text{M}$  TDZ treatment as early as day 9 (Fig. 4). Similar structures were observed in 30  $\mu\text{M}$  dicamba + 4  $\mu\text{M}$  and 30  $\mu\text{M}$  dicamba + 2  $\mu\text{M}$  TDZ combinations at later stages (days 12 and 15), indicating a dose-dependent response. The observed features, such as isodiametric cell clusters and yellowish-green pigmentation, aligned with known markers of embryogenic callus (Silveira *et al.* 2013; Woo *et al.* 2021).

These results affirm that the TTC assay, supported by morphology and microscopy, offers reliable and non-destructive methods for identifying embryogenic callus. Although the TTC colorimetric assay alone effectively reflects cell viability and metabolic activity, it cannot distinguish embryogenic from non-embryogenic tissues. Nevertheless, it serves as a useful preliminary screening tool for selecting viable cells to be further examined microscopically. Therefore, microscopic observation remains indispensable for confirming the presence of callus with embryogenic potential. The optimized hormonal combination of 30  $\mu\text{M}$  dicamba and 8  $\mu\text{M}$  TDZ is therefore recommended for the efficient establishment of embryogenic suspension cultures in *P. bullata*, a woody medicinal plant with significant biotechnological value.



**Fig. 7.** Formation of yellowish-green globular calli in the treatment of the combination of 30  $\mu\text{M}$  dicamba and 8  $\mu\text{M}$  TDZ

**Table 1.** Morphology of Cell Suspension Cultures for 18 days

	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18
<b>0 <math>\mu\text{M}</math> MSO</b>	Light yellowish	Yellowish	Light yellowish brown	Light yellowish brown	Yellowish brown	Dark yellowish brown
<b>30 <math>\mu\text{M}</math> Dic</b>	Light yellowish	Yellowish	Yellowish	Light yellowish brown	Yellowish brown	Yellowish brown
<b>2 <math>\mu\text{M}</math> TDZ</b>	Light yellowish	Yellowish	Light yellowish brown	Light yellowish brown	Yellowish brown	Yellowish brown
<b>4 <math>\mu\text{M}</math> TDZ</b>	Light yellowish	Yellowish	Light yellowish brown	Light yellowish brown	Yellowish brown	Yellowish brown
<b>6 <math>\mu\text{M}</math> TDZ</b>	Light yellowish	Yellowish	Light yellowish brown	Light yellowish brown	Yellowish brown	Yellowish brown
<b>8 <math>\mu\text{M}</math> TDZ</b>	Light yellowish	Yellowish	Light yellowish	Light yellowish brown	Yellowish brown	Yellowish brown
<b>10 <math>\mu\text{M}</math> TDZ</b>	Light yellowish	Yellowish	Light yellowish brown	Light yellowish brown	Yellowish brown	Yellowish brown
<b>30 <math>\mu\text{M}</math> Dic + 2 <math>\mu\text{M}</math> TDZ</b>	Light yellowish	Yellowish	Yellowish	Yellowish	Yellowish green	Light yellowish brown
<b>30 <math>\mu\text{M}</math> Dic + 4 <math>\mu\text{M}</math> TDZ</b>	Light yellowish	Yellowish	Yellowish	Yellowish green	Yellowish green	Light yellowish brown
<b>30 <math>\mu\text{M}</math> Dic + 6 <math>\mu\text{M}</math> TDZ</b>	Light yellowish	Yellowish	Yellowish	Yellowish	Yellowish	Light yellowish brown
<b>30 <math>\mu\text{M}</math> Dic + 8 <math>\mu\text{M}</math> TDZ</b>	Light yellowish	Yellowish	Yellowish green	Yellowish green	Yellowish green	Light yellowish brown
<b>30 <math>\mu\text{M}</math> Dic + 10 <math>\mu\text{M}</math> TDZ</b>	Light yellowish	Yellowish	Yellowish	Yellowish	Light yellowish brown	Yellowish brown

**Table 2.** Colour Changes of *P. bullata* Suspension Cells After TTC Assay

	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18
<b>0 <math>\mu\text{M}</math> MSO</b>	Yellowish	Yellowish	Yellowish	Yellowish	Brown	Dark brown
<b>30 <math>\mu\text{M}</math> Dic</b>	Light pink	Light pink	Yellowish	Yellowish	Brown	Dark brown
<b>2 <math>\mu\text{M}</math> TDZ</b>	Light pink	Light pink	Yellowish	Light pink	Light pink	Brown
<b>4 <math>\mu\text{M}</math> TDZ</b>	Light pink	Yellowish	Light pink	Yellowish	Brown	Brown
<b>6 <math>\mu\text{M}</math> TDZ</b>	Light pink	Yellowish	Yellowish	Yellowish	Brown	Brown
<b>8 <math>\mu\text{M}</math> TDZ</b>	Light pink	Yellowish	Light pink	Yellowish	Brown	Brown
<b>10 <math>\mu\text{M}</math> TDZ</b>	Light pink	Yellowish	Yellowish	Yellowish	Brown	Brown
<b>30 <math>\mu\text{M}</math> Dic + 2 <math>\mu\text{M}</math> TDZ</b>	Light pink	Yellowish	Pink	Pink	Red	Light pink
<b>30 <math>\mu\text{M}</math> Dic + 4 <math>\mu\text{M}</math> TDZ</b>	Light pink	Light pink	Pink	Pink	Red	Light pink
<b>30 <math>\mu\text{M}</math> Dic + 6 <math>\mu\text{M}</math> TDZ</b>	Light pink	Light pink	Pink	Light pink	Light pink	Light pink
<b>30 <math>\mu\text{M}</math> Dic + 8 <math>\mu\text{M}</math> TDZ</b>	Light pink	Light pink	Pink	Red	Red	Pink
<b>30 <math>\mu\text{M}</math> Dic + 10 <math>\mu\text{M}</math> TDZ</b>	Light pink	Yellowish	Light pink	Pink	Light pink	Light pink

## CONCLUSIONS

1. The combination of 30  $\mu\text{M}$  dicamba and 8  $\mu\text{M}$  thidiazuron (TDZ) produced the highest dry weight ( $0.087 \pm 0.025$  g) and distinct yellowish green callus, indicating strong embryogenic potential.
2. Microscopic analysis detected globular embryogenic structures as early as day 9, validating the effectiveness of the hormonal treatment in inducing somatic embryogenesis.
3. A rapid and cost-effective triphenyl tetrazolium chloride (TTC) colorimetric assay was successfully established to identify embryogenic callus in *P. bullata* cell suspension cultures.
4. The TTC staining yielded intense red coloration, confirming high cellular viability and metabolic activity in embryogenic callus.
5. The integration of TTC-based viability testing and morphological screening provided a practical and scalable alternative to conventional histological and molecular techniques.
6. The developed method enhances tissue culture protocols for *P. bullata*, supporting efficient propagation and conservation of this recalcitrant woody medicinal species.

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