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Histochemistry: Indicator for somatic embryogenesis of oil palm (*Elaeis guineensis* Jacq.) genotypes Pisifera and Tenera

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Abstract

Somatic embryogenesis plays an important role in oil palm clone regeneration by enabling large-scale propagation of genetically uniform planting materials. Nevertheless, the practical implementation of this technique remains challenging, particularly due to the low efficiency of callus induction and embryos formation. This study aimed to evaluate the morphological characteristics and histochemical profiles associated with embryogenic development during oil palm somatic embryogenesis. The materials used consisted of callus and somatic embryos derived from Pisifera and Tenera genotypes with La Mé genetic background, each represented by four replications. Histochemical analyses were carried out using iodine potassium iodide (IKI) solution for starch detection, Millon's reagent for protein localization, and potassium dichromate for phenolic compounds, followed by semi-quantitative intensity scoring on a 0-3 scale. Morphological observations identified four callus types, namely *compact, aqueous, nodular,* and *aggregated*, while embryo developmental progressed thorough *globular, heart* and *scutellar* phases. Histochemical analysis revealed that clone P1 showed the highest starch and protein accumulation (1.87) with relatively low phenolic content (1.22). In contrast, clone P2 exhibited the highest phenolic intensity (1.87) and the lowest starch accumulation (0.71). Clones P3 and P4 displayed relatively balanced metabolite accumulation patterns (1.58-1.87), suggesting more stable physiological condition during somatic embryo development. Two-way ANOVA demonstrated significant effect of metabolite type (starch, phenolics, and protein) and developmental phase (P1- P4) on staining intensity, with a significant interaction between both factors ($P<0.0001$). Overall, these findings indicate that the histochemical profiles reflect the physiological status of tissues and may provide preliminary insights into embryogenic development during oil palm somatic embryogenesis.

[Keywords: embryogenic competence, histo-chemistry, oil palm, somatic embryogenesis, tissue culture]

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is one of Indonesia's major plantation commodities, playing a vital role in the national economy as a primary source of vegetable oil. The increasing demand for palm oil both domestically and internationally, highlights the need for superior and high-quality planting material, as productivity in oil palm cultivation is largely dependent on the quality of the planting stock used. (Corley & Tinker, 2016; Dirjenbun, 2024). Therefore, the development of efficient and reliable propagation techniques is crucial.

A relevant analytical approach for this purpose is histochemistry, which allows the examination of the content and distribution of biochemical compounds within callus tissues. Through histochemical techniques, metabolic changes during embryogenesis, including the accumulation of starch, proteins, lipids, and phenolics, can be visualized and associated with physiological characteristics of embryogenic tissues (Fehér, 2015; Silva et al., 2016; Panggabean et al., 2021). Given these considerations, histochemical evaluation may provide preliminary insights into physiological responses associated with somatic embryogenesis. This study aimed to analyze the morphological characteristics and histochemical profiles of callus and embryos during the early phase of somatic embryogenesis. The findings are expected to provide fundamental insights to may support future studies on the optimization of oil palm regeneration methods for oil palm through somatic embryogenesis.

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Material and Methods

Plant materials

Research was conducted at the Plant Tissue Culture Laboratory, Indonesian Oil Palm Research Institute (IOPRI), North Sumatra, Indonesia. The plant materials consisted of in vitro cultures of callus and somatic embryos of oil palm with a La Mé genetic background, four genotypes were used: P1 and P2 (Pisifera, ± 20 months in culture) and P3 and P4 (Tenera, ± 39 months in culture).

Morphological observation and histochemical staining

Morphological observations were conducted by randomly selecting in vitro oil palm callus and embryos from four culture flasks per genotype, with four replications. Samples of callus and embryos were subsequently examined using a stereomicroscope (Olympus SZX10). Histochemical analyses were performed using three specific staining reagents: 1% iodine for starch detection, Millon's reagent for protein localization, and 5% potassium dichromate (K_2CrO_4) for phenolic compounds. Observations were conducted under a binocular microscope (Olympus DP23).

a) Starch test: Fresh callus sections were soaked in 50% ethanol for 3 min, stained with 1% iodine, covered with a glass slide, and gently heating at 45°C (Jensen, 1962).

b) Protein test: Millon's reagent was applied directly to the tissue, and positive reactions were indicated by reddish-brown coloration due to the presence of tyrosine (Handayani, 2017).

c) Phenolic test: Tissues were immersed in 5% potassium dichromate for 30 min, rinsed with distilled water, and mounted with glycerin. The presence of phenolic compounds was indicated by dark yellowish-brown coloration (Gabe, 1968).

Intensity assessment

Metabolite accumulations were analyzed based on staining intensity using semi-quantitative scale. The two parameters: presence (0 = absent, 1 = present) and intensity (+++ = high [3], ++ = medium [2], + = low [1], – = none [0]). The average intensity values of starch, phenol, and protein for each genotype were calculated and visualized as bar graphs.

Statistical analysis

The intensity data were analyzed using Two-Way ANOVA ($\alpha = 0.05$) to evaluate the effects of developmental phase (P1-P4), metabolite type (starch, phenolics, protein), and their interactions. Post hoc analyses were performed using Tukey's HSD test for pairwise comparison. Statistical analyses were conducted using GraphPad Prism version 10.

Results and Discussion

Morphological characteristics of oil palm callus under in vitro

In general, throughout the oil palm tissue culture process, the developing explants exhibit diverse characteristics and responses. The morphological characteristics of callus derived from the Pisifera type of oil palm are presented in Figure 1. Based on the morphological observation presented in Figure 1, the oil palm callus exhibited variations in structure and color that can serve as indicators of embryogenic potential. The callus types A3, B1, and B3 demonstrated typical characteristics of embryogenic callus, including a bright appearance (whitish-yellow to cream), compact structure, slightly friable texture, and aggregated form (irregular clumps of cell masses). The B2 (aqueous) callus was characterized by a glossy surface, watery and fragile texture, and a translucent white to pale- yellow coloration. These characteristics are consistent with the findings of Silva et al. (2016) who reported that embryogenic callus is generally compact or slightly friable, bright in color, and possesses globular structures. Moreover, Gomes et al. (2017) stated that, during the early stages of development, cell masses are typically yellowish in color, less watery in appearance, compact in structure, and relatively small in size. Conversely, callus types A1 and A2 tended to be non-embryogenic. A1 appeared compact and brownish, indicating necrotic regions with an uneven friable texture. A2 exhibited an irregular nodular morphology, with the presence of brown tissue. Dark or blackish coloration commonly indicated phenolic oxidation or tissue stress (Yelnititis, 2013). Furthermore, Saptari (2018) emphasized that the type of explants plays a crucial role in determining the success of callus initiation in in vitro culture. The higher the meristematic activity of the cell or tissues used, the greater their capacity to undergo dedifferentiation and form callus induced by plant growth regulators (PGRs).

Morphological characteristics of oil palm embryos under in vitro.

The subsequent development stage of oil palm embryogenesis following embryogenic callus formation in the somatic embryogenesis phase, leading to embryo differentiation. At this stage, somatic embryos exhibit distinct morphological characteristics, which can be identified through microscopic observation to determine their development phases. These embryos are generally from secondary callus that has been previously

proliferated in embryo induction media. The developmental stages of somatic embryos are characterized by variation in morphology, ranging from spherical to slightly elongated shapes, with whitish coloration and a watery texture (Ilham et al., 2024). The analysis of morphological characteristics of callus, such as color, texture, and shape, serves as an important indicator of embryogenic potential and thus plays a vital role in determining the success of plant regeneration through tissue culture. The morphological characteristics of somatic embryos of Tenera-type oil palm at various developmental stages are presented in Figure 2.

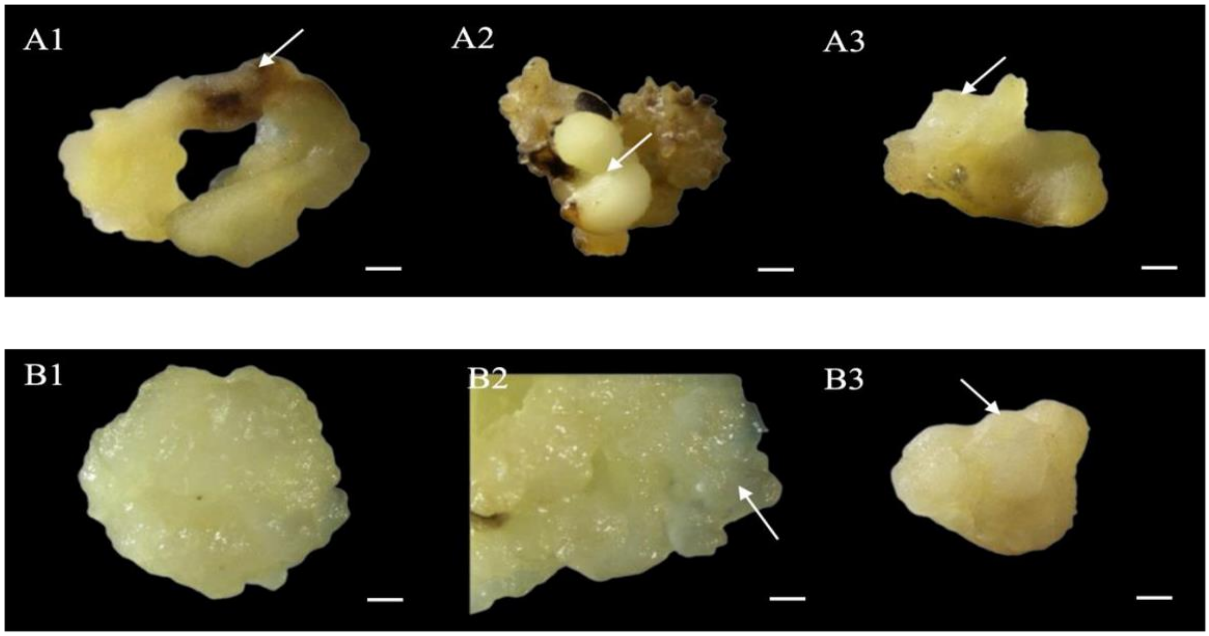

Figure 1. Morphological characteristics of oil palm callus type P1 (A) and P2 (B); A1) compact (non-embryogenic), A2) nodular, A3) aggregate, B1) compact (non-embryogenic), B2) aqueous, B3) aggregate. Bar = 5 mm. (Arrows indicate the morphology of each callus developmental phase observed).

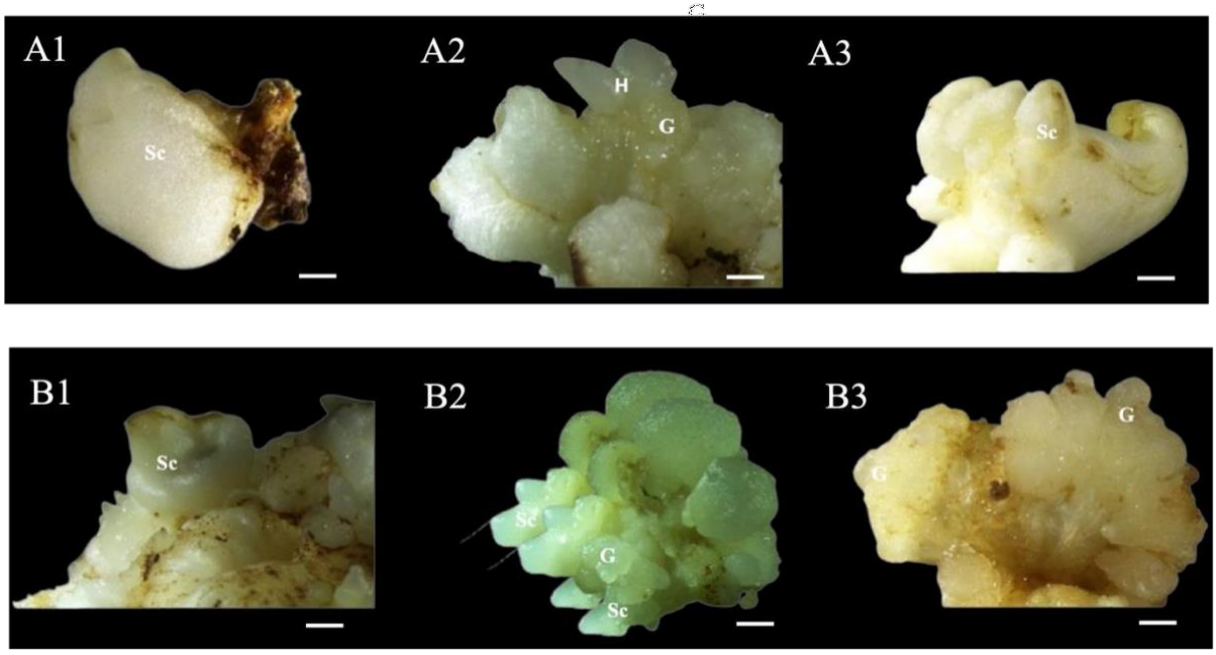

Figure 2. Morphological characteristic of oil palm embryos P3 (A) and P4 (B); A1) scutellar (Sc), A2) heart (H), A3) scutellar (Sc), B1) scutellar (Sc), B2) scutellar (Sc) and globular (G), and B3) globular (G). Bar = 5 mm.

Somatic embryogenesis in oil palm is one of the key vegetative propagation techniques used for plant multiplication. The morphological development of embryos in P3 and P4 exhibited differences that reflect the ongoing somatic embryogenesis process. At stage P3 (A), the embryos begin to display more defined structure. In figure A1, the scutellar (Sc) structure indicates the initiation of distinctive monocot organ formation. Subsequently, Figure A2 shows the emergence of Heart (H) and Globular (G) forms, demonstrating active cellular differentiation. In Figure A3, the scutellar (Sc) structure becomes more pronounced, indicating further embryo development. Morphological characteristics at stage P4 (B) show that the scutellar forms are still visible in B1 and B2; however, multiple globular (G) structures are also observed in B2, indicating that somatic embryo development is still ongoing. Figure B3 is dominated by the globular form, which may suggest delayed differentiation or the proliferation of undifferentiated embryonic masses. The presence of different embryo shapes (scutellar, heart, and globular) within a single developmental stage indicates that somatic embryogenesis in oil palm does not occur synchronously. This is consistent with the findings of Ibrahim (2015), who reported that the success of somatic embryogenesis is strongly influenced by genetic factors, media composition, and the application of PGRs. Morphological variation among treatments may also indicate differences in responses to culture media and environmental conditions.

Histochemical analysis as a physiological indicator in somatic embryogenesis.

Histochemical staining revealed distinct differences in the distribution and accumulation of starch, phenolics compound, and proteins among the developmental phases (Figure 3). These metabolites play key roles in energy storage, stress response, and cellular differentiation, making their relative intensities useful indicators of embryogenic competence. P1 tissues showed the highest starch and protein intensities, both reaching approximately ±1.87, while phenolic intensity remained relatively low (±1.22). This indicates that P1 callus is in a metabolically active state, characterized by the accumulation of energy reserves and nitrogenous compounds, yet with moderate phenolic activity. High starch content in callus tissue is generally associated with developmental progression, including induction, proliferation, and regeneration stages (Puad et al., 2024). Conversely, P2 callus exhibited a different pattern, where phenolic intensity was the highest (±1.87), while starch intensity was the lowest (±0.71). Increased phenolic accumulation in P2 may be associated with stress responses or physiological imbalances that inhibit the success of somatic embryogenesis. However, several studies have also reported that embryogenic cells may accumulate higher phenolic content compared to non- embryogenic cells, depending on the type and concentration of the compounds involved (Meguellati et al., 2022). This suggests a dualistic role of phenolics in embryogenesis, where a balanced concentration can promote differentiation, while excessive accumulation may exert toxic or inhibitory effects. In the embryonic phases (P3 & P4), starch intensity remained high (±1.87), whereas phenolics and proteins showed relatively balanced levels (±1.58). This pattern implies that embryonic tissues have reached a more stable physiological condition and metabolic maturity. Somatic embryo regeneration is commonly associated with reduced phenolic content and increased peroxidase activity, which mitigates the toxic effects of phenolics (Pancaningtyas, 2015).

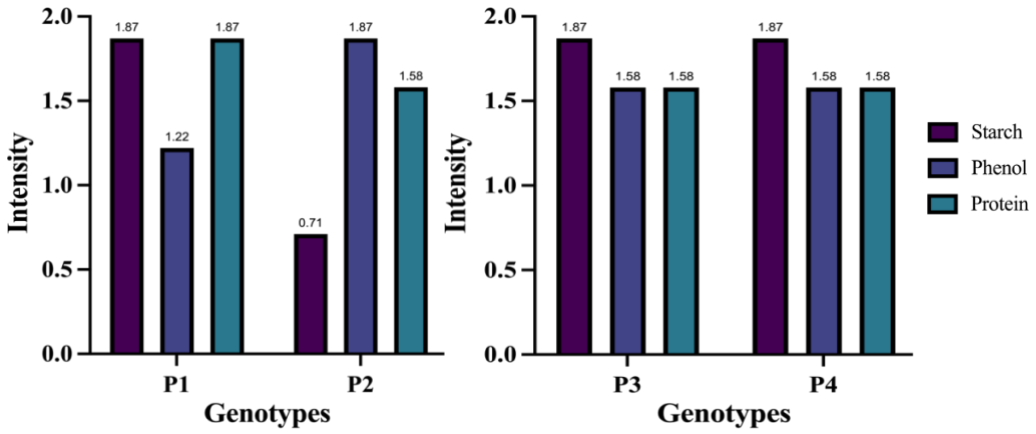

Figure 3. Intensities of starch, phenol compounds, and proteins in the callus (P1 & P2) and embryo (P3 & P4) developmental phases of oil palm tissues. Statistical analysis using Two-Way ANOVA revealed significant differences among developmental phases, metabolite types, as well as their interaction ($P<0.0001$).

The Two-Way ANOVA results showed that both metabolite type (starch, phenolics, protein) and developmental phase (P1-P4) had a significant effect on staining intensity ($P<0.0001$), with a significant interaction between the two factors. This indicates that variations in staining intensity were influenced by the combined effects of developmental stage (callus and embryo) and metabolite type (starch, phenolics, and protein). Based on the mean trend, starch intensity tended to increase in the embryonic phase compared to the callus phase, whereas phenolics and proteins remained relatively stable during embryonic development. Tukey's HSD post hoc test did not reveal any significant pairwise differences. This may be associated with limited sample size, variability among biological replicates, and overlapping staining intensity values between developmental phases. In addition, the interpretation of these statistical results should be approached cautiously because the evaluated developmental phases also differed in genotypes and culture duration, which may collectively contribute to the observed variation in staining intensity. These results suggest the presence of dynamic metabolic changes during the transition from callus to embryo development. Therefore, histochemical staining may provide preliminary physiological insights associated with somatic embryogenesis in oil palm tissues. Moreover, these findings support future strategies for culture medium optimization and selection of initial explants for somatic embryogenesis studies.

The visualization of metabolite distribution through histochemical staining in oil palm callus is presented in Figure 4, which supports semi-quantitative data and clarifies the spatial distribution of each metabolite at the tissue level. Figure 4 illustrates the localization of starch (A1, B1), phenolic compounds (A2, B2), and proteins (A3, B3) in callus derived from two different oil palm clones. These differences provide preliminary indications of metabolic activity and the regenerative potential of each callus type. The distribution of starch, phenolics, and protein in callus tissues serves as an early biochemical indicator that reflects physiological activity differences among clones, ultimately determining their ability to grow and regenerate under tissue culture conditions.

Histochemical staining using iodine-potassium iodide (IKI) solution showed starch accumulation, indicated by the presence of dark-colored granules within the callus tissue (A1 and B1). Callus from clone P1 (A1) exhibited a more uniform distribution of starch, suggesting differences in physiological status and embryogenic potential between clones. Starch is a key energy reserve required during the early stage of somatic embryogenesis. The high starch content of the P1 callus reflects active metabolic processes and cellular readiness for division and differentiation. During the proliferation phase, callus tissue accumulates sugars, particularly glucose and starch, as energy reserves for the subsequent regeneration phase. This accumulation plays an essential role in supporting the high energy demand during organogenesis and helps maintain osmotic balance in the culture medium by regulating soluble sugar level (Efferth, 2019). Carbon storage activity in the form of starch also serves as an indicator of stable physiological integrity, suggesting that cells are not experiencing oxidative stress (Fehér, 2015). In crops such as oil palm, starch accumulation in callus cells has been associated with higher embryogenic potential and more stable cell viability.

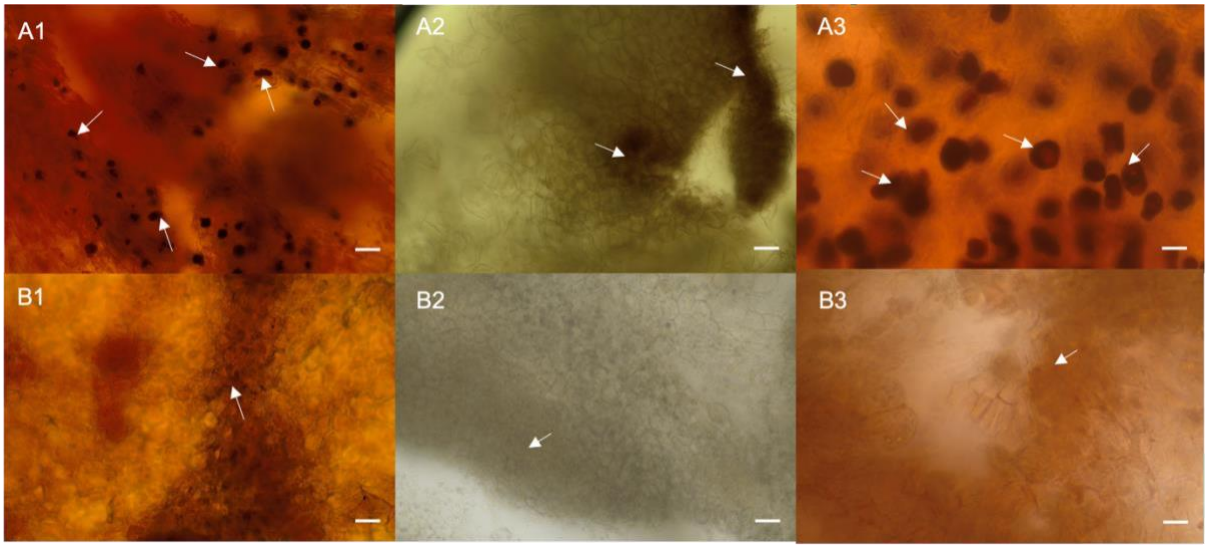

Figure 4. Histochemical analysis of oil palm callus P1 (A) and P2 (B); A1) starch, A2) phenolics, A3) protein, B1) starch, B2) phenolics, B3) protein. Scale bar: A1 = 100 µm, A2 = 100 µm, A3 = 50 µm, B1 = 100 µm, B2 = 100 µm, B3 = 50 µm.

Therefore, histochemical staining for starch detection can be used as an important parameter to evaluate callus readiness to enter the somatic embryogenesis phase.

Histochemical staining using potassium dichromate (K_2CrO_4) revealed the presence of phenolic compounds, indicated by brown to dark-yellow coloration. According to Argyropoulou et al. (2010) observation with fluorescence microscopy combined with potassium dichromate staining confirms phenolic accumulation, as the intensity of the stain corresponds to phenol presence within the tissue. Callus from clone P1 (A2) exhibited relatively low phenolic accumulation, while P2 (B2) showed higher and more uniformly distributed phenolics. High levels of phenolic compounds are generally associated with stress responses and tissue browning, which may inhibit somatic embryogenesis (Fehér, 2015). Excess phenolics can lead to oxidative reactions and the formation of reactive oxygen species (ROS), reducing cell viability and lowering callus differentiation capacity. In addition, elevated soluble phenolic content such as naringenin and cinnamic acid has been reported to suppress somatic embryo development by inducing early lignification or disrupting the regulation of developmental gene expression (Zein El-Din et al., 2021). Therefore, the lower accumulation of phenolics observed in P1 indicates a more stable physiological status and a more favorable environment for embryo development.

Histochemical staining with Millon's reagent confirmed the presence of proteins, indicated by reddish to reddish-brown coloration consistent with a positive Millon's reaction. Callus from P1 (A3) exhibited higher protein accumulation and more uniformity than P2 (B3). Elevated protein content reflects active metabolic and biosynthetic processes that are essential for the establishment and maintenance of embryonic structures during somatic embryogenesis (Radoeva et al., 2020). Proteins play fundamental roles in regulating gene expression, hormonal signalling, and cell differentiation, ultimately supporting the formation of structurally complete embryos (Khadem et al., 2023). Thus, the substantial protein abundance observed in P1 suggests a higher embryonic potential compared with P2. In contrast, the biosynthesis of phenolic compounds in plants is governed by the phenylpropanoid pathway, involving key enzymatic steps mediated by Phenylalanine Ammonia-Lyase (PAL), Cinnamate-4-Hydroxylase (C4H), 4-Coumarate: CoA Logase (4CL), and Chalcone Synthase (CHS). PAL catalyses the conversion of phenylalanine to cinnamic acid, which is subsequently hydroxylated byC4H to p-coumaric acid. The latter is activated by 4CL to form p-coumaroyl-CoA, a central precursor for lignin, flavonoid, and other phenolic derivatives. CHS initiates the branch toward flavonoid biosynthesis by converting p-coumaroyl- CoA into chalcone. The activity of these enzymes is strongly influenced by genetic factors and tissue physiological status. Variations in the expression of PAL, C4H, 4CL, and CHS among clones may therefore lead to differential phenolic accumulation in callus tissue, affecting tissue viability, the extent of oxidative stress, and the capacity of cellular differentiation and somatic embryogenesis (Biała & Jasiński, 2018; Weckx et al., 2019; Xin et al., 2022). The visualization of metabolite distribution through histochemical staining in oil palm embryos clone P3 (A) and P4 (B) is presented in Figure 5.

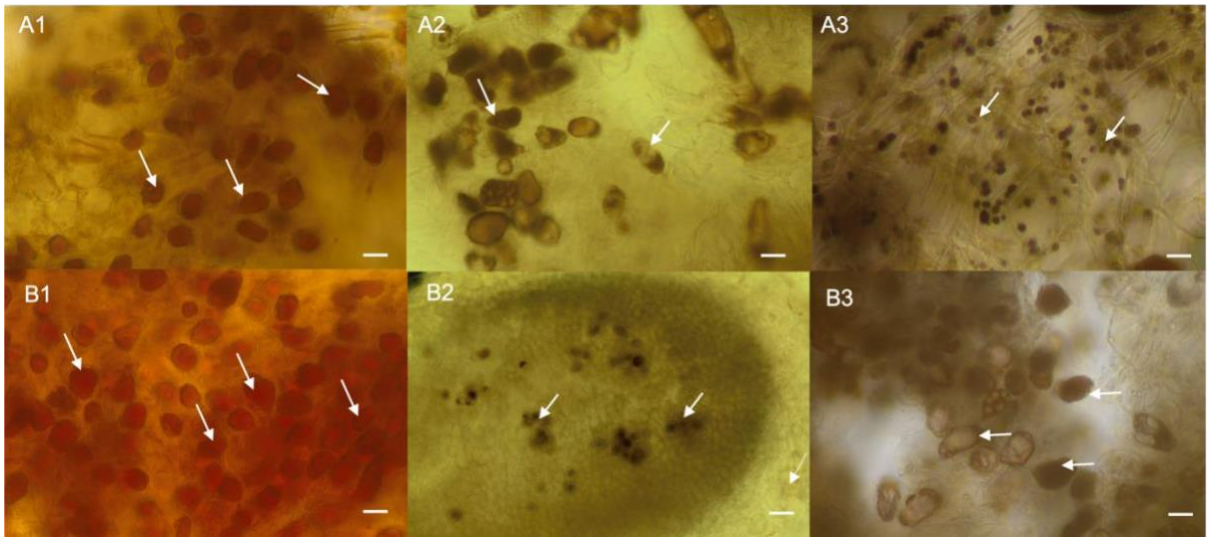

Figure 5. Histochemical localization in somatic embryos of oil palm P3 (A) and P4 (B); A1) starch, A2) phenolics, A3) protein, B1) starch, B2) phenolics, B3) protein. Scale bars: A1 = 50 µm, A2 = 50 µm, A3 = 50 µm, B1 = 50 µm, B2 = 100 µm, B3 = 50 µm.

Histochemical analysis of somatic embryos from P3 and P4 revealed distinct physiological characteristics in the accumulations and spatial distribution of key metabolites, including starch, phenolic compounds, and proteins. Both P3 and P4 exhibited strong starch accumulation (A1, B1), indicating active carbohydrate synthesis and storage to support somatic embryogenesis development. Starch serves as a major vigor reserve required during cell division and differentiation, consistent with earlier reports that highlight its critical role during somatic embryogenesis (da Silva et al., 2015). The elevated starch levels suggest that both P3 and P4 possess stable metabolic capacity to sustain embryo maturation. Moreover, previous findings also reported a positive correlation between starch accumulation and high embryogenic competence in oil palm tissues (Pàdua et al., 2018).

Moderate phenolic accumulation observed in P3 and P4 (A2, B2) indicates an active stress-response mechanism, as phenolics in in vitro culture are typically associated with wound-induced stress, oxidative reactions, or hormonal imbalance. These compounds act as cellular antioxidants and protective agents. However, excessive accumulation can trigger tissue browning and inhibit regeneration. Liu et al. (2024) reported that high phenolic content, particularly in species prone to phenolic oxidation, leads to browning through oxidative reactions that suppress growth and morphogenesis. This issue can be mitigated by incorporating antioxidants such as ascorbic acid or polyvinylpyrrolidone (PVP) into the culture system (Amente & Chimdessa, 2021).

Protein staining demonstrated uneven distribution in both P3 and P4 (A3, B3), suggesting potential limitations in protein synthesis or turnover, or suboptimal nitrogen availability in the culture medium. Efficient nitrogen metabolism is essential for successful regeneration because nitrogen supports the biosynthesis of structural and enzymatic proteins required for cell differentiation and proliferation. Hamdeni et al. (2022) confirmed that optimal nitrogen metabolism ensures adequate protein provision for tissue development, whereas disruptions may impair morphogenic responses due to insufficient production of key regulatory protein.

This study provided preliminary insights into physiological characteristics associated with embryogenic development. However, differences in genotype, developmental stage, and culture duration among the evaluated materials may collectively influence the observed histochemical responses. Therefore, the present findings should be interpreted as preliminary observations rather than definitive indicators of embryogenic competence. Moreover, integrating histochemical evaluation with molecular and physiological approaches could provide a more comprehensive framework for understanding the regulatory mechanisms underlying somatic embryogenesis and improving tissue culture efficiency in oil palm.

Conclusion

This study characterized the morphological features and histochemical profiles of oil palm callus and somatic embryos. Embryogenic callus generally exhibited bright coloration, compact to slightly friable texture, nodular structures, and aggregated cell masses, while somatic embryos developed through *globular*, *heart,* and *scutellar* stages. Histochemical observations showed that starch, protein, and phenolic distributions varied among developmental phases and may reflect differences in tissue physiological status. High starch and protein accumulation combined with relatively lower phenolic content were associated with tissues showing embryogenic characteristics. Histochemical evaluation may also assist tissue culture laboratories in monitoring physiological responses during callus and embryo development, thereby supporting culture medium optimization and explant selection. Further studies integrating regeneration performance, synchronized developmental stages, and molecular markers are necessary to validate the predictive value of these histochemical traits.

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