Journal of Tropical Crop Science Vol. 11 No. 3, October 2024
www.j-tropical-crops.com

Received 17/07/2024; Revised 27/09/2024; Accepted 22/10/2024
https://doi.org/10.29244/jtcs.11.03.307-319

Histological Analysis of Somatic Embryogenesis from Immature Zygotic Embryo of Wild Banana *Musa acuminata* ssp. *malaccensis*

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Abstract

Somatic embryogenesis, a crucial plant regeneration method, has become indispensable for crop improvement, particularly for species reliant on somatic cell manipulation techniques. Optimization of this process necessitates an understanding of the developmental stages involved. This study investigates the histological aspects of somatic embryogenesis in *Musa acuminata* ssp. *malaccensis* derived from immature zygotic embryos. Through detailed histological analysis, we aimed to elucidate the morphological changes and cellular organization occurring during the various stages of somatic embryogenesis, from induction, culture proliferation, and somatic embryo development to plantlet conversion. The initial stages of embryogenesis, characterized by nodules, were primarily composed of meristematic cells with high cell division activity. These cells contained tetrad-like structures that could develop into distinct two- and four-celled proembryoids or proembryogenic aggregates. Our histo-anatomical analysis revealed that embryogenic cultures proliferated through multiple pathways simultaneously: somatic embryo budding, proembryo formation, and pro-embryonic mass formation from both internal and peripheral cells. At the stage of somatic embryo development, embryos with a well-defined protoderm layer, containing cells with prominent nuclei and dense cytoplasm, potentially regenerate into plantlets. Furthermore, histological examination revealed the presence of procambium within mature somatic embryos, which subsequently developed into the vascular system of the complete plantlet.

Keywords: embryogenic culture, histo-anatomy, meristematic cells, pro-embryonic masses, protoderm, tetrad-like cells

Introduction

Banana (*Musa* spp.) is a staple food crop for millions worldwide, yet its cultivation faces challenges including disease susceptibility, abiotic stresses, and limited genetic diversity. Indonesia is recognized as a primary center of origin and diversity for *M. acuminata*, both wild and cultivated banana (Nasution, 1991; Poerba et al., 2019). Wild banana species, particularly *Musa acuminata*, offer a rich source of genetic variation for improvement. Wild bananas and their relatives are increasingly used in pre-breeding due to their potential for disease resistance, tolerance to abiotic stress, and other desirable traits (Pilley and Tripathi, 2007; Tingnam et al., 2023). Notably, the *malaccensis* subspecies of *M. acuminata* is a significant contributor to cultivated bananas and is widely found in Indonesia and Malaysia (De Langhe et al., 2009; Ahmad et al., 2020). These wild bananas can be integrated into the banana breeding programs via conventional breeding, or through biotechnological approaches such as somatic hybridization, gene transfer and more recently gene editing. These biotechnological approaches require the availability of cell to plant regeneration systems, and the most efficient one is regeneration via somatic embryogenesis.

Somatic embryogenesis, an important plant regeneration method, has become an indispensable tool for biotechnology-based crop improvement, such as somatic hybridization, genetic transformation, and

Received 17/07/2024; Revised 27/09/2024; Accepted 22/10/2024
https://doi.org/10.29244/jtcs.11.03.307-319

gene editing (Adero et al., 2023) that particularly rely on single cell event. In banana, this regeneration pathway has been known to be genetic dependent and limited to certain genome, inefficient and therefore still need improvement (Justine et al., 2022). Optimization of this process may require a deeper understanding of the developmental stages involved. Plant regeneration including somatic embryogenesis relies on cellular totipotency, which allows competent somatic cells to undergo a series of transformations, ultimately forming embryos with the potential to develop into whole plants (Bidabadi and Jain, 2020). Somatic embryogenesis may occur directly from explant or indirectly after the formation of a callus stage; these processes are known as direct and indirect somatic embryogenesis, respectively (Sivanesan et al., 2022). Previous studies have hypothesized that both processes are extremes of one continuous developmental pathway wherein callus represents a reprogramming step of unorganized tissue that precedes embryo formation (Rocha et al., 2016).

Histological and scanning electron microscopy observations have proven valuable for determining the cellular composition and structures of tissues at different embryogenesis stages. Embryogenic cultures and non-embryogenic callus exhibit distinct morphological features, such as color and texture. Notably, embryogenic callus contains somatic embryos at various developmental stages (Borji et al., 2018). This morpho-histological analysis is employed to confirm and study the morphology of cells, allowing us to distinguish embryogenic cells from those that fail to respond, aiding in the differentiation between SE-specific structures and organogenic structures, and enhancing our understanding of plant cell totipotency and morpho-histological patterns.

The study of morpho-histology patterns is essential for understanding the impact of various conditions on cellular and embryonic development (de Araújo Silva-Cardoso et al., 2020). Histological confirmation of embryogenesis in bananas has been previously reported to strengthen studies on regeneration pathway in in vitro culture (Grapin et al., 1996; Navarro et al., 1997; Jalil et al., 2008; Jafari et al., 2015). These studies reveal similar patterns of somatic embryo development: the active division of meristematic cells (induction), formation of meristematic centers (globular compact callus or pro-embryogenic cell clumps), and conversion of meristematic or pro-embryogenic cells into embryogenic cells (Navarro et al., 1997). To fully understand the in vitro development of wild *Musa* through somatic embryogenesis, further research is needed on the specific stages of embryogenesis. Elucidating these stages could improve regeneration rates and enhance the applications of wild *Musa* in biotechnology research. Anatomical and histological studies of somatic embryogenesis in wild bananas can significantly refine tissue culture protocols by identifying precise stages of embryo development and distinguishing true embryogenic cells from non-embryogenic tissues. This prevents unnecessary subculturing and enhances the efficiency of the embryogenesis process (Fehér, 2019; Grapin et al., 1996).

This paper presents a comprehensive anatomical-histological analysis of immature zygotic embryo-derived somatic embryogenesis in *Musa acuminata* ssp. *malaccensis*, starting from culture induction, culture proliferation, somatic embryo development, and plant conversion on semi-solid medium. This information will contribute to refining somatic embryogenesis protocols for wild bananas, ultimately facilitating their integration into banana breeding programs.

Material and Methods

Plant Materials

The cluster of mother plants *Musa acuminata* ssp. *malaccensis* was cultivated in the 'Demplot 04' within the Ir. Sukarno Science and Technology Area at the National Research and Innovation Agency in Cibinong, Bogor, Indonesia. Immature zygotic embryos were extracted from fruits of the wild banana cluster as explants and prepared using the method developed by Handayani et al. (2024).

Induction and Proliferation of Embryogenic Cultures

Immature zygotic embryos (IZEs), 56 to 63 days after anthesis (at about 80 to 90% fruit maturity), were excised from sterilized seeds under a laminar airflow cabinet. Seed surface sterilization involved two 10-minute immersions in 96% ethanol. Fifteen IZEs were then cultured on Petri dishes (90 x 15 mm) containing 25 mL induction medium designed to promote somatic embryogenesis. This induction medium is composed of MS-based formulation (Murashige and Skoog, 1962) with modification according to Strosse et al. (2003) and Escobedo-Gracia Medrano et al. (2016) and also by incorporating some adjustments. The medium consisted of the following components (in $mg.L^{-1}$): Macro and micro salts based on the MS medium formulation with a modified NH_4NO_3 (1200) and KNO_3 (3030) (Witjaksono and Litz, 1999a), standard concentration of MS micro salts including FeNaEDTA (36.7) and vitamins, Biotin (1.00), PVP (MW 360,000) (10.0),

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L-Glutamine (100), Casein hydrolysate (250), Proline (50), sucrose (30.000), 2,4-Dichlorophenoxyacetic acid (1.0), and Gelrite® (2000) (Handayani et al., 2024). The pH of all media was adjusted to 5.8, and after dissolving the gelling agent, the media were sterilized by autoclaving for 20 minutes at 121°C and 15 psi.

After 8-12 weeks, explants exhibiting new growth were transferred to the proliferation medium. This medium was identical to the induction medium, except the macronutrients were used at half strength concentration and supplemented with 1.0 $mg.L^{-1}$ 2,4-D (2,4-Dichlorophenoxyacetic acid) and 1.0 $mg.L^{-1}$ picloram (Handayani et al., 2024). During subculture, cultures with good proliferation, particularly those exhibiting a friable whitish yellowish granular-nodular morphology, were selected for further use. Highly proliferating cultures were subculture to fresh medium every 6–8 weeks, for further multiplication.

Cultures of induction embryogenesis were placed in plastic boxes to provide darkness at a room temperature of 22 ± 2°C until the explants started to form new growth: unorganized structures or embryogenic cultures. The embryogenic cultures subsequently developed were maintained under the same conditions as those used for induction.

Somatic Embryo Development

Embryogenic cultures exhibiting friable granular-nodular structure were transferred to a somatic embryo development medium (25 mL) in Petri dishes (90 x 15 mm). This somatic embryo development medium shared the same basal composition as the induction media but was devoid of plant growth regulator and with modifications of the sucrose concentration, with an increased sucrose concentration of 40 $g.L^{-1}$ and the addition of 3 $mg.L^{-1}$ Gelrite®. After eight weeks of incubation, opaque somatic embryos (SEs) were transferred to a plant conversion medium. This medium had the same composition as the somatic embryo development medium except that it contained the same concentration of Gelrite® (3 $mg.L^{-1}$) but with a reduced sucrose concentration of 30 $g.L^{-1}$. The plant conversion medium was supplemented with plant growth regulator of 0.5 $mg.L^{-1}$ BA (benzyl adenine) and 0.5 $mg.L^{-1}$ GA_3 (gibberellin) (Handayani et al., 2024). Cultures were initially maintained in darkness for two weeks, followed by exposure to light intensity of 9.76-12.20 $\mu mol.m^{-2}.s^{-1}$ for 16 hours under the same temperature previously used.

Morphological Analysis

Morphological observations were conducted using a Nikon SMZ1000 stereo microscope (Nikon, Japan) to assess the characteristics of both the initial zygotic embryo (IZE) and any new growth arising from the explants and subsequent growth and development that arose from the inoculum. The magnification used for observation ranged from approximately 0.8 to 4.0 × 10 magnification. Distinct morphological features developed during induction and proliferation stages, including whitish friable granular-nodular structures, yellowish friable granular-nodular structures, compact dome nodular structures, and spongy-unorganized callus, were documented. The morphology of somatic embryos, including opaque, transparent (hyperhydric), and subsequent sprouting or germinated somatic embryos, was also carefully documented.

Histological Analysis

To identify the true nature of the structures developed during somatic embryogenesis (callus, nodular-globular structures, proembryos, or somatic embryos), cultures exhibiting visually distinct morphologies at each stage of development, including the immature zygotic embryos used as explants before induction treatment, were sampled for histological analysis. A modified paraffin method (Sass, 1951) with safranin and fast green staining was employed. Samples from each stage were fixed in FAA solution (formaldehyde, acetic acid, ethanol) for approximately 24 hours. Subsequently, they were gradually dehydrated in a series of solutions containing tert-butanol, absolute ethanol, and distilled water in specific combination ratios. The dehydrated samples were then embedded in paraffin with a solidification point of 56-58°C. Tissue sections 10– 2 µm thick were cut using a Leica rotary microtome and stained with 1% safranin and 2% fast green. Finally, the histologically stained sections were observed under a light microscope (Olympus BX53). These histological observations distinguished: a) callus: unorganized tissue composed of vacuolated parenchyma cells; b) pro-embryoids or pro-embryogenic masses (PEM): organized cluster of isodiametric, cytoplasmic-dense cells, delineated from surrounding tissue (may or may not have an epidermis); c) somatic embryo (SE): organized tissue composed of vacuolated or cytoplasmic-dense cells arranged in layers and enveloped by a distinct epidermis (globular or later stage). In this study, a granular-nodular structure refers to a globular, round structure, yellowish-white in color, dense, and composed of small isodiametric cells. These terms correspond to the visual descriptions provided by Schumann et al. (1995).

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Results

Histology Embryogenic Cultures on Induction Medium

Immature zygotic embryos of wild banana *M. acuminata* ssp. *malaccensis* ranges from 0.05 to 0.10 mm (Figure 1 A-1, A-2). When cultured in an induction medium containing either 2,4-D or picloram, immature zygotic embryo (IZE) explants exhibited various subsequent developmental responses: development of shoots (precocious germination), callus growth (soft, watery and translucent callus or compact, yellowish callus) (Figure 1 C-1), development of granular-nodular structures (globular and embryo-like structures) (Figure 1 B1, C1), or no-growth or died. The new growth formation began 6 to 12 weeks after the IZE explants were cultured on the induction medium.

Histological analysis of zygotic embryos was conducted to investigate the nature of the cellular basis of their embryonic tissue, which is the origin of the subsequent growth and development during somatic embryogenesis. This analysis revealed evidence of tissue differentiation as it pictures the presence of shoot meristem, root meristem, coleoptile tissue, vascular tissue (vessels), and cotyledon-scutellum-like tissue (Figure 1 A-3, A-4). Interestingly, those notable tissues of different structures and functions are composed mostly of cells with dense nuclei, isodiametric, and highly cytoplasmic. The difference among those tissues was the cell component's size, density, and level of vacuolation. For example, the shoot meristem tissue was constructed with a much smaller cell size and tightly spaced compared to parenchyma tissues with larger and less compactly spaced (Figure 1 A-3,4).

Induction of embryogenesis from explant immature zygotic embryo is marked by two stages, i.e., the formation of lobes or nodular structures (Figure 1 B-1) at the first stage after several subcultures some more proliferative granular-globular-nodular structures developed together with apparent somatic embryo-like structures Figure 1 C-1). At the first stage, upon anatomical analysis, the tissue of the original explants enlarges and proliferates to form the meristematic zones (Figure 1 B-2, B-3) is proembryos, pro-embryogenic masses somatic embryos, and somatic embryo masses (Figure 1 C-3).

Nodular or lobe structures were initially formed as clusters of lobes outgrowth measuring >1 mm (Figure 1 B-1). Six weeks after the subculture of this whole culture, many smaller structures with globular shapes of less than 1 mm developed (Figure 1 C-1). The aggregates of lobe structures formed during the induction stage exhibit clusters of meristematic cells. These meristematic cells are characterized by small and dense (black arrow), while the surrounding cells are larger and unorganized parenchyma cells (Figure 1 B-2, 1 B-3, yellow arrow). Highly meristematic cells are present within the nodular structures (Figure 1 B, C, black arrow). Morpho-histological observations reveal that cultures with a nodular morphology have meristematic cells located internally within the tissue, surrounded by callus tissue composed of parenchyma cells (Figure 1 B-3, C-2). These meristematic cells exhibited high division activity, with the potential to form discrete two- and four-celled proembryoids or initial proembryogenic aggregates (Figure 1 C-3, red arrow). Three friable granular structures developed six weeks after transferring the 12-week-old explants to a fresh medium of the same composition. These granular structures were smaller (0.5 – 1.0 mm) than the nodular structures (> 1.0 mm) and accompanied by profuse callus growth (Figure 1 C-1, red arrow). They appeared as isolated structures approximately 2 mm in diameter and were composed of meristematic cells surrounded by parenchyma cells (Figure 1 C-3).

Histology of Embryogenic Culture Proliferation

During the embryogenic proliferation stage, wild *Musa* cultures originating from IZE explants differentiated into four primary structures: (a) friable, whitish globular-nodular structures, (b) friable, yellowish granular-globular structures, (c) compact, whitish nodular structures, and (d) friable, irregular callus with a spongy-like texture (Figure 2). The distinction of those four primary structures from a single published report has not been found.

The friable, whitish globular structures had a round, smooth surface with sizes of about 1 mm, though smaller structures were also noticeable (Figure 2 A-1). Each round structure was not wholly spherical but sometimes composed of more than one lobe. Forceps can easily separate these structures. Anatomical and histological examination of these structures showed the presence of meristematic cell clusters enveloped by epidermis, arranged in a continuous series of lobes of different sizes ranging from 200 to 800 µm (Figure 2 A-2, black arrow), mounted onto core structures of round parenchymatous cells. These structures can be interpreted as embryo budding, multiple somatic embryos, or cleavage polyembryony. More detailed observation of other parts of the structures showed the formation of smaller somatic embryos; some became isolated, but most were still attached (Figure 2 A-3). Interestingly, inside an embryonic lobe (red arrow), many round meristematic cells of proembryos measuring 30 – 60 µm. Once they grew larger, an

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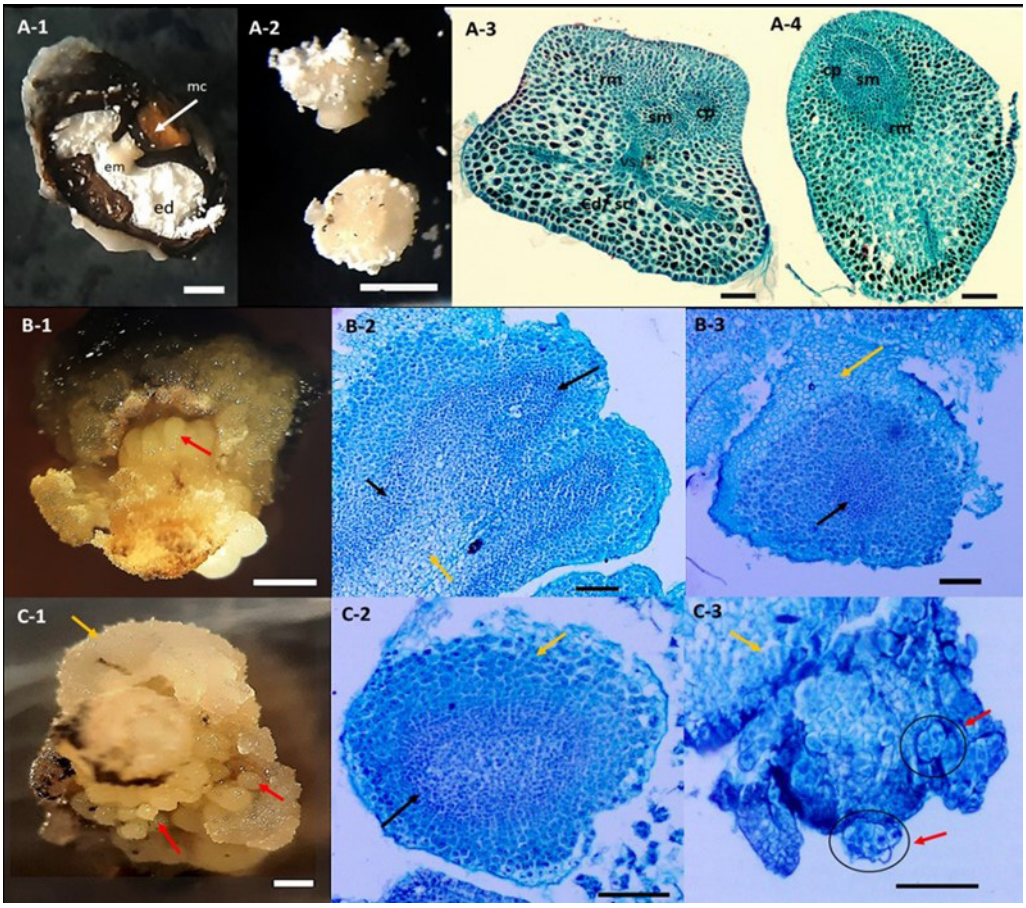

Figure 1. Morphology and histology of zygotic embryo explant of wild banana *M. acuminata* ssp *malaccensis* during culture induction stage. A-1) Longitudinal section of a seed (mc: micropyle, em: embryo, ed: powdery endosperm); A-2) isolated embryos with some powdery endosperm stuck to them; A-3) longitudinal section) of an embryo; A-4) Cross-section of an embryo showing shoot meristem (sm), root meristem (rm),: coleoptile (cp),: vessel (vs), cotyledon (cd), scutellum (sc); B-1) Callus and nodular structures developed from the immature embryos explanted on induction medium after 12 weeks; B-2) Histology of friable nodular callus on induction medium indicating branching of meristematic regions; B-3) Nodular structures consisted of meristematic cells. C-1) Friable granular structures with a size smaller than that of nodular structures and profuse callus developed from the explants, six weeks after transfer of the 12-week-old explant onto to fresh medium of the same composition; C-2) Histology of granular structures showed isolated structures with a size of about 2 mm in diameter and composed of meristematic cells surrounded by parenchyma cells. C-3) highly meristematic cells present at the peripheral nodular structure. Scale bars: A1,2; B1; C1 = 1 mm; A3,4; B2,3; C2,3 = 100 µm. The red arrow indicates embryogenic cells, the yellow arrow indicates non-embryogenic cells (parenchyma cells), and the black arrow indicates meristematic cells.

epidermis was formed (Figure 2 A-3). Therefore, it can be interpreted that in these structures, the cultures proliferated by cleavage polyembryony and the formation of proembryos that quickly develop into embryos, either internally or peripherally, of the somatic embryos.

The friable, yellowish granular-globular structures (Figure 2 B-1) have distinct friable structures as they are easily separable by forceps, and the composing structures have round shapes. The surface of this round shape did not look smooth but relatively grainy. The histo-anatomy observation indicated the presence of cells with dense cytoplasm, isodiametric with prominent nuclei organized as proembryos (round, circles), and somatic embryo budding (red arrow) covering the whole sample tissue in a very dense fashion (Figure 2 B-2). Careful observation of the tissue sample also indicated actively dividing cells in the 2-cell or tetrad stage cells, indicating the presence of proembryo initials (circles) (Figure 2 B-3, red arrow). Therefore, it can be interpreted that this culture was highly proliferating by forming mostly proembryos and proembryonic masses, and

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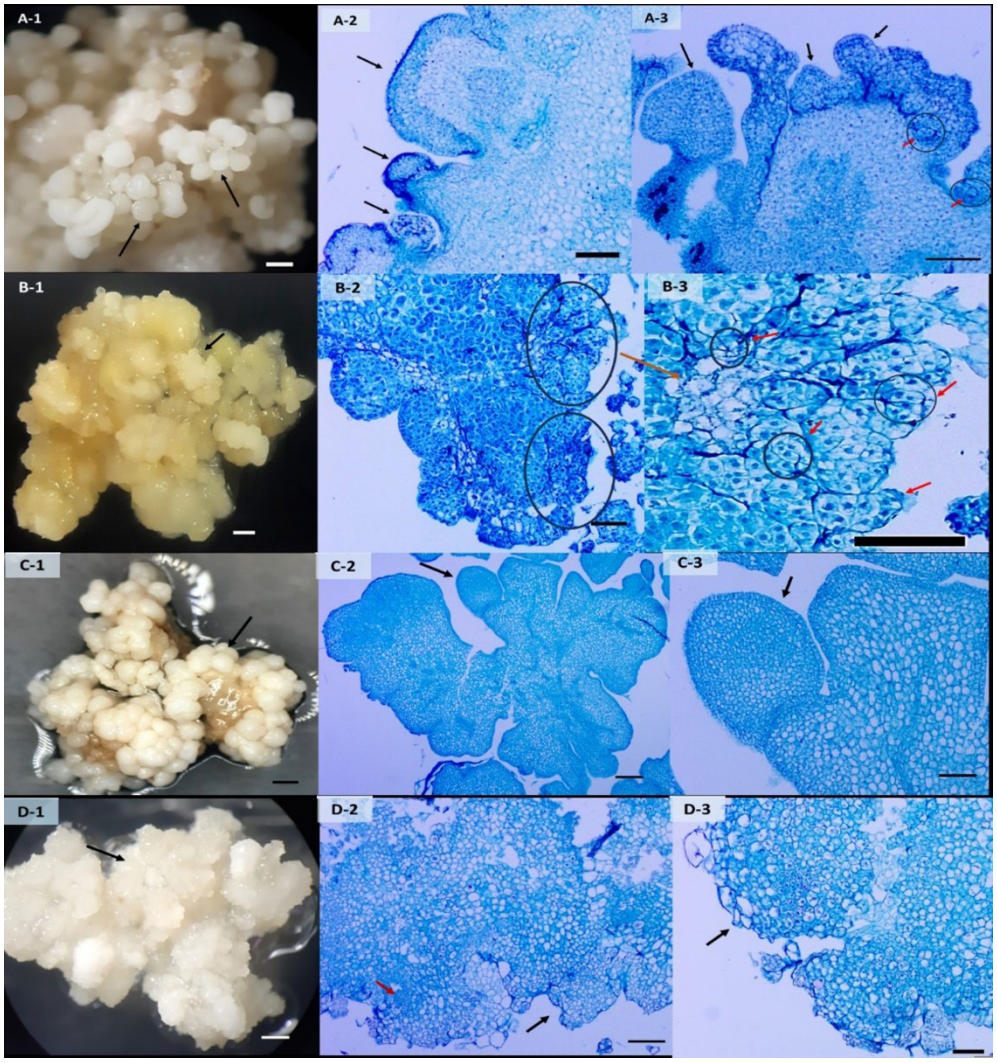

Figure 2. Morphology and histology of proliferating embryogenic culture of wild banana *M. acuminata* ssp *malaccensis*. A-1) whitish friable granular structures, with the size of 1 mm or less; A-2) granular structures showed organ of embryos as they are enveloped by epidermis and proembryos of smaller size of 100 um or less; A-3) small size (less than 200 um) of embryos (indicated by epidermis) and proembryos (with no epidermis) at the surface of the structures; B-1) yellowish friable granular structures; B-2) proembryonic masses (PEMs) as indicated by organized meristematic cells and part of somatic embryos as indicated by the presence of epidermis densely populated the sample tissues internally or peripherally; B-3) tetrad-like cells as proembryo initials are evident in the tissue indicating highly proliferating cultures; C-1) compact nodular culture; C-2) somatic embryo budding or cleavage embryony; C-3) somatic embryo budding is characterized by the present of the epidermis enveloping the whole surface of the structures; D-1) unorganized/cottony/spongy callus; D-2) cluster of meristematic cells but do not form highly organized structures; D-3) meristem-like cells scattered among highly vacuolated parenchymatous cells; bar A-1, B-1, C-1, D-1 = 1 mm; bar A-2, B-2, C-2, D-2 = 200 µM; bar A-3, B-3, C-3, D-3 = 100 µM. The black arrow in Figures A and C indicates the embryos with epidermis, the black arrow in Figure D shows disorganized cells, and the red arrow indicates pro-embryogenic cells.

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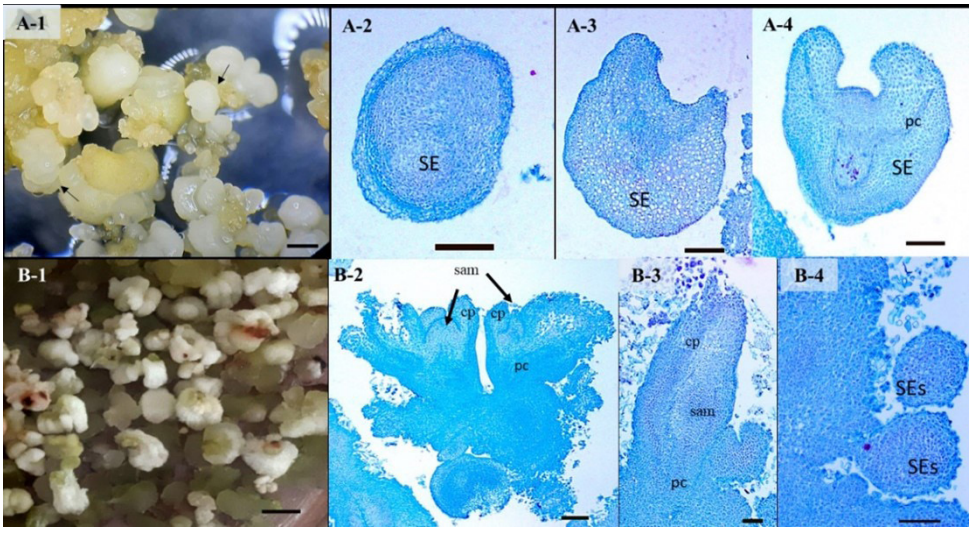

Figure 3. Morphology and histology of somatic embryo development and sprouting. A-1) Opaque somatic embryos developed on somatic embryo development medium; A-2) The globular somatic embryo is delineated by epidermis; A-3) Somatic embryo with the presence of apical meristem, A-4) Histology of bipolar somatic embryo with apical and basal meristems and scutellum and procambium; B-1) Somatic embryo sprouting indicated by the protuberance emerges from SE (blue arrow); B-2) Histology of sprouting SE, the protuberance gradually elongates and differentiates into a rudimentary shoot structure; B-3) Histology of sprouting SE, the apical meristem leading to the elongation and branching of the shoot; B-4) Histology of secondary SE; epidermis (ep); shoot apical meristem (sam). Scale bars: A-1, B-1: 1 mm; A-2,3,4: 100 µm; B-2,3,4: 200 µm.

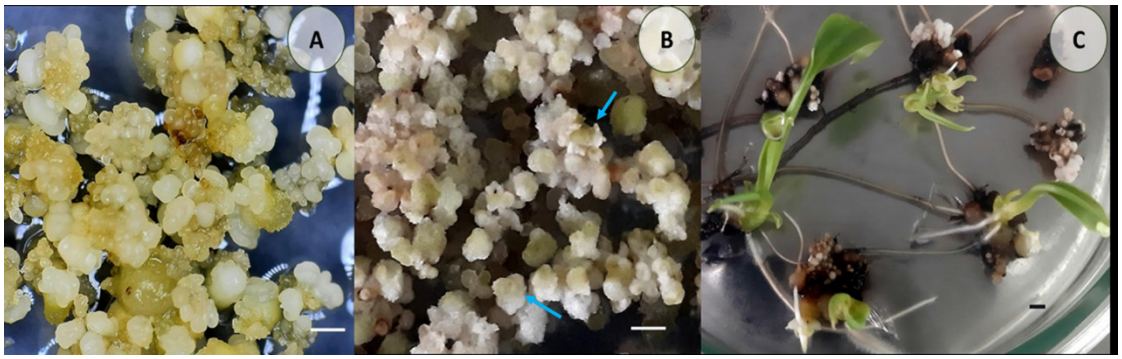

Figure 4. Regeneration of somatic embryogenesis of wild *Musa*, A) Opaque of somatic embryos (SEs); B) germination of SEs (blue arrow); C) SEs conversion to plantlets. Scale bar = 1 mm

individual somatic embryos were not evidence. The compact, whitish nodular structures (Figure 2 C-1) were composed of dome-shaped structures attached to a core tissue, with each dome or cluster of domes having 1–3 mm diameters. The whole structure of the dome was not composed of just 1–2 big lobes; instead, there were many lobes of different sizes, all of which had surfaces covered with epidermis. The presence of epidermis indicated that this structure was an organ—an embryo undergoing budding or cleavage embryony. The budding of embryos in this kind of structure was massive, covering the whole structure, which differed from the previously described structure in Fig. 2 A-1, 2, 3, where budding occurs scarcely. A closer examination of this compact nodular structure of somatic embryo budding showed that the buds eventually detached from the core by forming a nick enveloped by epidermis and released somatic embryos. Therefore, in these embryogenic structures, the culture proliferation was somatic embryo proliferation by cleavage embryogeny.

The last type of structure formed in embryogenic cultures was friable and irregular, with a spongy-like texture (Figure 2 D-1). This culture was soft and composed of delicate tissues easily separable with forceps. The morphology seemed composed of noticeable units, but the units had no definite shape. Anatomy and histology across that tissue revealed cells in the isodiametric stage; some were

Received 17/07/2024; Revised 27/09/2024; Accepted 22/10/2024
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isodiametric with low-density cytoplasm. These cells formed some organization, but not enough to be called organs (Figure 2 D-2, D-3).

Histology of Somatic Embryo Development and Plant Conversion

After eight weeks in the somatic embryo development medium, the inoculum formed somatic embryos that were opaque in color and measured about 1-2 mm in diameter (Figure 3 A-1). Histologically, the white opaque structures were somatic embryos since they were delineated with protoderm and epidermis (Figure 3 A-2). They generally had a round structure even though some developed a cotyledon-like structure with meristem between them, which was supposed to be scutellum (Figure 3 A-3). The somatic embryos formed bipolar structures with meristematic cells at both ends and flanked by procambium (Figure 3 A-4). When the somatic embryo started to germinate or sprout, the scutella tissue, shoot apical meristem, and root apical meristems differentiated distinctly from the rest of the tissue (Figure 3B-1). The presence of meristematic cells, parenchymal cells, protoderm, procambium, and a shoot apical meristem supports the identification of shoot formation. The apical meristem gives rise to new cells, leading to the elongation and branching of the shoot, while the formation of procambium (pc), the precursor to vascular tissues (Figure 3 B-2,3). Secondary somatic embryos could also develop from the germinating somatic embryos indicating the high somatic embryogenesis capacity of the inoculum (Figure 3 B-4).

Somatic embryos (SEs) can develop into either opaque or transparent (hyper-hydric) embryos (Figure 4-A). Following successful sprouting, plantlets develop shoots and roots (Figure 4C). Opaque SEs, typically derived from friable nodular-granular structures, regenerate into normal plantlets more readily (Figure 4). Conversely, hyper-hydric embryos have a significantly higher rate of failing to form shoots. However, some SEs may only form shoots or roots, and some may fail to germinate.

Discussion

We have presented complete histo-anatomical evidence of somatic embryogenesis from the explant of immature zygotic embryos of wild *M. acuminata* ssp. *malaccensis*. The somatic embryogenesis process that we presented covers all the stages of growth on the semi-solid media, including culture induction, culture proliferation, somatic embryo development, and somatic embryo sprouting as an early stage of plant conversion.

Two stages mark the induction of embryogenesis from explant immature zygotic embryos: the formation of lobes or nodular structures (Figure 1B-1) at the first stage, followed by the development of more proliferative granular-globular-nodular structures along with apparent somatic embryo-like structures after several subcultures (Figure 1C-1). At the first stage, anatomical analysis showed that the tissue of the original explants enlarged and proliferated to form meristematic zones (Figure 1B-2, B-3), which are proembryos, proembryonic masses, somatic embryos, and somatic embryo masses (Figure 1C-3). The development of round globular nodular structures on the surface of enlarging explants during induction is expected in the somatic embryogenic system in bananas (Ponni et al., 2019). These round structures are called callus, whether globular or nodular in shape, friable in texture, and yellow or white (López et al., 2022). This so-called nodular embryogenic callus has been reported to develop from explants of globular stage zygotic embryos of ssp. *malaccensis* (Escobedo-Gracia Medrano et al., 2014), showing that it develops into proembryonic masses, which later develop into various somatic embryos at different stages of development. During induction, different structures such as yellow nodular callus, white compact callus, and nodular non-embryogenic callus are distinguishable from the embryogenic callus of date palm (*Phoenix dactylifera* L.) (Zein El Din et al., 2021). The embryogenic cells produce proembryos and somatic embryos, which develop into somatic embryos and their germinations (Debbarma et al., 2019). Thus, the identification of the embryogenic nature of the callus is determined based on empirical evidence of somatic embryo germination.

The development of proembryos and somatic embryos from explant immature zygotic embryos may be considered direct and indirect embryogenesis by cloning existing embryonic cells. In direct embryogenesis, embryos are formed directly from explants without a callus formation phase. Examples of this phenomenon can be seen in somatic embryogenesis from zygotic embryos of *Canna* spp. (Gan et al., 2023), and *Camellia oleifera* (Zhang et al., 2021). Meanwhile, indirect embryogenesis involves the formation of somatic embryos through an intermediate callus phase, which is common in banana embryogenesis when using immature male flowers as explants (Ardhani et al., 2024).

The development of proembryos or somatic embryos, as indicated by the histo-anatomical evidence we presented in a somatic embryogenesis system, is morphologically recognized as nodular, friable, and embryogenic cells, as reported by Grapin et al. (1996), Sidha et al. (2007), and Rustagi et al.

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https://doi.org/10.29244/jtcs.11.03.307-319

(2019). This is generally identified as an embryogenic callus. However, identifying such friable nodular, globular, and granular structures as embryogenic callus may not be entirely appropriate since they are not true callus. Callus can be briefly described as unorganized growing tissue represented by a mass of dedifferentiated cells (Fehér, 2019). Nevertheless, callus in bananas, which develops into embryogenic culture, has been reported to be induced from somatic tissue, such as meristematic domes, as described by Strosse et al. (2003).

During the proliferation of this embryogenic culture, there were four distinct types of morphology: i.e., friable whitish nodular to globular structure, friable yellowish granular-globular structure, compact nodular structure, and spongy cottony callus. Nodular structures appear small, compact, and often irregularly shaped bumps or nodules. They are not spherical but resemble 'knots' or clumps. In contrast, globular structures are more rounded, smooth, and spherical, resembling an early-stage embryo and representing a more organized structure compared to nodular forms. The growth of four distinct types of structures that represent different modes of proliferation (proembryo and somatic embryo, dominant proembryos, somatic embryo budding, and dedifferentiation of somatic embryos or proembryos) at the same time has not been reported elsewhere. Each of these four types of cultures was reported to be represented by one embryogenic suspension culture of one subspecies of *M. acuminata* Colla (Handayani et al., 2024); this morphology was also reported by Debbarma et al. (2019) from an embryogenesis culture of a cultivated banana from the immature male flower. Different cultivar proliferated and represented by different type of culture was reported in avocado (Witjaksono et al., 1999b) in which some cultivars showed embryogenic cultures dominated by proembryonic masses, some dominated by somatic embryo type, a mixture type dominates some.

Embryogenesis is a natural and spontaneous process of plant regeneration started by the syngamy for the formation of a zygote followed by unequal division and division in multiplane of anticlinal and periclinal but in an organized pattern to form proembryos, followed by differentiation of protoderm or epidermis to form globular embryos, scutellum and differentiation of meristem covering shoot -root axis, vasculature, and other tissues such as haustorium in case of the palm of the monocot (Juarez-Escobar et al., 2021). Upon germination, the embryo grows root and shoots and develops into a normal plant. Somatic embryogenesis of wild bananas initiated from an immature zygotic embryo essentially mimics normal development with some deviations as described as follows. During induction, the remnant of embryogenic cells in the immature embryo explant underwent cloning due to auxin in the medium. On one side, the embryogenic cell continues to proliferate, but the natural and spontaneous development of the embryonic cell gives rise to proembryo and embryo continues to take effect (Carneros et al., 2023). The relative strength of each of those two factors: the exogenous auxin and internal development force, dictate the results whether it is proembryo or proembryonic masses (friable granular structures), mix of somatic embryo and proembryos, somatic embryo budding (compact nodular structures) or dedifferentiated embryogenic masses/cells (cottony or spongy callus) ((Williams and Maheswaran, 1986; Li et al., 2022).

Histological analysis of developing somatic embryos revealed the presence of a surrounding layer of protoderm (pr) cells. This protoderm layer, the first identifiable tissue layer during somatic embryogenesis, stains densely and surrounds the meristematic embryogenic cells. Its presence is a hallmark somatic embryo development feature (Thorpe and Stasolla, 2001; Rustagi et al., 2019). As development progresses beyond the late globular stage, characterized by the establishment of bipolarity (Borji et al., 2018), further differentiation becomes evident. Studies by Jafari et al. (2015) indicated that only embryos with a distinct protoderm layer, containing cells with prominent nuclei and dense cytoplasm, could regenerate into plantlets. This is followed by the formation of the apical meristem (shoot bud), root apical meristems, and procambium (vascular tissue precursor) (Sugimoto et al., 2019).

Based on morphological and anatomical analysis from the induction process to the germination stage of somatic embryos, our findings suggest that embryogenesis in wild *Musa* from immature zygotic embryos can occur directly through embryo budding or cleavage polyembryony, including the development of tetrad-like cells into proembryoids. Anatomical analysis revealed that embryogenic cultures developed through several concurrent pathways: somatic embryo budding, proembryo formation, and pro-embryonic mass formation from internal and peripheral cells (Williams and Maheswaran, 1986). These pathways were also observed in the proliferation of somatic embryogenesis of subspecies *malaccensis* using embryogenic cell suspension culture (Handayani et al., 2024).

Upon transfer of the embryogenic cultures to a medium without auxin, somatic embryos develop, indicating that a natural and spontaneous process occurs for embryo development as it does for zygotic embryo development. The fact that somatic embryos

Received 17/07/2024; Revised 27/09/2024; Accepted 22/10/2024
https://doi.org/10.29244/jtcs.11.03.307-319

eventually germinate indicates normal development parallel to zygotic embryos, even though additional growth regulators such as cytokinin improve somatic embryo germination.

Conclusion

We have provided clear evidence that somatic embryogenesis initiated from immature zygotic embryos generates friable, granular-nodular structures comprising proembryos, proembryonic masses, somatic embryos, somatic embryonic masses, and some dedifferentiated somatic cells that form callus. Selective subculturing of these granular-nodular structures, excluding callus tissue, enables efficient proliferation of the embryogenic culture. This highly prolific system simultaneously employs multiple pathways, including forming embryo initials within and on the periphery of existing proembryonic masses (PEMs). These initials develop into proembryos, which can enlarge and repeatedly form new PEMs. Concurrently, some proembryos differentiate into somatic embryos, while others give rise to somatic embryo budding. This dynamic process persists in the presence of auxin. After removing auxin and under elevated osmotic conditions with a firmer medium, proembryos develop normally into mature, opaque somatic embryos germinating under suitable conditions. This anatomical-histological analysis enhances our understanding of somatic embryogenesis in *Musa acuminata*, helping to optimize culture conditions and protocols. It can also serve as a reference for studies on the developmental differences of somatic embryos and is a crucial resource for advancing research in plant tissue culture and biotechnology.

Acknowledgment

This paper is part of Tri Handayani's (TH) PhD dissertation. The work was partially funded by the Research Organization for Life Sciences and Environment under BRIN's DIPA Rumah Program for 2022-2023 with TH as the principal investigator. TH received a degree from a research scholarship from BRIN between 2019 and 2022. We thank Dr. Yuyu S. Poerba and Dr. Fajarudin Ahmad for providing the genetic materials for this research; the genetic materials were accession that has been screened for resistance against banana bunchy top virus under the project of BBTV Mitigation: Community Management in Nigeria, and Screening Wild Banana Progenitors for Resistance funded by Bill and Melinda Gates Foundation as Investment ID OPP1130226, later revised to project ID INV-010652, administered by The University of Queensland, Australia.

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