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An Efficient Somatic Embryogenesis and Plant Regeneration from Immature Embryo of Wild Banana *Musa acuminata* ssp. *malaccensis*

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Abstract

Wild banana *Musa acuminata* ssp. *malaccensis*, an ancestor of cultivated bananas, possesses valuable genetic diversity, including resistance genes to fusarium wilt, and demonstrates high environmental adaptability. These traits are important for pre-breeding programs, whether by conventional breeding, which is a lengthy process, or by taking advantage of somatic cell manipulation techniques such as somatic hybridization, which requires an efficient plant regeneration system like somatic embryogenesis. We have established an efficient and comprehensive protocol for somatic embryogenesis of this wild Musa using immature zygotic embryo explants covering culture induction and proliferation, somatic embryo development, and subsequent plant conversion. Embryogenic culture was induced on a complex-modified MS medium supplemented with 1 $mg.L^{-1}$ 2,4-D auxin or 5 $mg.L^{-1}$ picloram. The embryogenic cultures proliferated in the form of granular or nodular structures, which was best obtained by reducing the picloram concentration to 1 $mg.L^{-1}$ and combining it with the same concentration of 2,4-D at a half-strength macro-nutrient of basal medium. Embryo development from embryogenic cultures and regeneration into shoots. Proembryos as granular structures spontaneously matured into early-stage somatic embryos upon withdrawal of the strong auxin inducer. Increasing the sucrose and gelling agent concentrations in the growth medium improved somatic embryo formation from embryogenic cultures. The frequency of shoot formation from developed somatic embryos was increased by incorporating 0.5 $mg.L^{-1}$ BA and 0.5 $mg.L^{-1}$ GA_3 into the regeneration medium.

Keywords: embryogenic induction, medium, proliferation, somatic embryos, plant conversion

Introduction

Musa acuminata ssp. *malaccensis*, a wild banana subspecies, has played an important role in the genetics and establishment of cultivated bananas, contributing to their domestication (Perrier et al., 2009; Jeensae et al., 2021). This subspecies is known to resist fusarium wilt (*Fusarium oxysporum* f.sp. *cubense*), as observed in segregated populations (Ahmad et al., 2020). Notably, this subspecies served as the source for the reference genome for banana plants, the Double Haploid 'Pahang' (D'hont et al., 2012). Additionally, ssp. *malaccensis* exhibits resistance to black leaf streak and moderate resistance to BBTV disease (Rahayuniati and Subandiyah, 2022). The RGA2 gene, isolated from ssp. *malaccensis*, has been successfully incorporated into genetically engineered Cavendish lines, leading to increased resistance against fusarium wilt Tropical Race 4 (TR4) (Dale et al., 2017).

The development of new banana varieties through conventional breeding is hampered by several factors inherent to cultivated bananas, including seedless due to sterility, low fertility, and parthenocarpy, heterozygosity, and varying ploidy levels (Wang et al., 2021). Additionally, the process is time- and resource-intensive, requiring significant labor. For wild bananas, seeds are set when there is a natural crossing, even though limitations may arise from chromosome structure differences among some subspecies (Ahmad et al., 2021). However, the

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segregation of wild traits together with the desirable traits may require several crossings that take time.

Genetic improvement through somatic cell manipulation, including protoplast fusion/somatic hybridization, anther culture, genetic engineering for transgenics, or genome editing, is a viable alternative to conventional breeding. Somatic hybridization, for instance, offers a viable alternative for combining desirable traits from wild bananas into cultivated varieties by complementation. Somatic cell approaches, such as protoplast fusion, provide a means to combine traits from selected parents that may be difficult to achieve with natural crosses (Sarma et al., 2023). Protoplast fusion, in particular, enables the generation of tetraploid plants without the need for antimitotic compound induction, a crucial aspect for obtaining the desired triploid hybrids (Grosser and Gmitter, 2011; Kim et al., 2020). However, it necessitates a plant regeneration protocol via the somatic embryogenesis pathway (Xiao et al., 2009). In cultivated bananas, somatic embryogenesis has been established using various explants or inoculum, such as shoot-derived scalps (Sholi et al., 2009), split shoot tips (Uma et al., 2021), immature female flowers (Grapin et al., 2000), or immature male flowers (Natarajan et al., 2020). However, these existing protocols are often lengthy, tedious, inefficient, difficult to replicate, and exhibit low regeneration efficiency. Plant regeneration via somatic embryogenesis has also been reported in wild Musa and cultivated banana relatives using immature embryo explants for various species, including *M. acuminata* ssp. *microcarpa*, *M. balbisiana* (Escalant and Teisson 1989), *Ensete superbum* (Roxb.) Cheesman (Ponni and Nair, 2019), *M. acuminata* ssp. *burmannica* (Uma et al., 2012), and ssp. *malaccensis* (Escobedo-Gracia-Medrano et al., 2014). These reports are, however, preliminary, lacking comprehensiveness of viable protocols.

This paper reports an efficient protocol for the entire somatic embryogenesis process in *Musa acuminata* ssp. *malaccensis*, encompassing induction, proliferation, embryo development, and plant conversion from immature zygotic embryos. This study contributes to refining somatic embryogenesis protocols for wild bananas, ultimately benefiting future applications of wild Musa in biotechnology research.

Material and Methods

Plant Materials and Embryo Extraction

The plant materials were zygotic embryos isolated from wild banana *Musa acuminata* ssp. *malaccensis,* cultivated in the 'Demplot 04' of Ir. Sukarno Science and Technology Area, National Research and Innovation Agency, Cibinong, Bogor, Indonesia. Seeds were obtained from fruits harvested at a specific age and maturity, washed with running tap water, and promptly transferred to a laminar airflow cabinet for further treatment.

Basal Medium

The basal medium employed for embryonic culture induction and proliferation modified the medium described by Strosse et al. (2003) and Escobedo-Gracia Medrano et al. (2016). The basal medium was based on the MS (Murashige and Skoog, 1962) formulation and contained the following components: macro salts MS, micro salts MS including FeNaEDTA (36.70), and vitamins MS. This basal medium was further supplemented with Biotin (1.00 $mg.L^{-1}$), PVP (MW 360,000) (10.0 $mg.L^{-1}$), L-glutamine (100 $mg.L^{-1}$), casein hydrolysate (250 $mg.L^{-1}$), proline (5 $mg.L^{-1}$) and sucrose 3% (w/v). The medium was adjusted to pH 5.6 – 5.8 using 1 N HCl or NaOH before adding 2 $g.L^{-1}$ Gelrite® (Duchefa). After dissolving the gelling agent, the media were sterilized by autoclaving for 20 minutes at 121°C and 15 psi. Subsequently, 25 mL of media was added into sterile glass Petri dishes measuring 90 mm x 15 mm in a laminar airflow cabinet.

The treatment medium for embryogenic culture proliferation, somatic embryo development, and plant conversion was prepared as above, with some components modified according to treatments. Additionally, for the experiment comparing the concentration of NH_4NO_3 and KNO_3, the concentration of these two salts was adjusted according to the treatments. The concentration of the macro and micro-salts remained the same as that of standard MS.

Culture Environment

Cultures for inducing somatic embryogenesis were kept in opaque plastic boxes at a room temperature of 22 ± 2°C until the explants began to exhibit new growth, such as unorganized structures or embryogenic cultures. The developing embryogenic cultures were maintained under the same conditions as those used for induction. The light intensity for cultures exposed to light ranged from 9.76 to 12.20 $\mu mol.m^{-2}$ per sec, with a 16-hour photoperiod.

Induction of Embryogenic Cultures

Several sets of experiments were carried out to investigate the effects of some factors on embryogenic culture induction:

a) Effect of the mature and immature zygotic embryo:

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mature zygotic embryos (MZEs) were extracted from tree-ripe fruit harvested 70 – 84 days after anthesis. Meanwhile, immature zygotic embryos were harvested from fruit 56 to 63 days after anthesis (80 to 90% maturity level). The basal medium was enriched with 1.0 mg.L^{-1} 2,4-D.

b) Effect of growth regulator concentration: various concentrations of 2,4-D (0.0, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, and 10.00 mg.L^{-1}) and picloram (0.0, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, and 10.00 mg.L^{-1}) were tested in the basal medium.

In each experiment, 15 zygotic embryos were explanted onto individual Petri dishes, which served as replicates. Each treatment had 6 to 10 replicates arranged and analyzed in a randomized complete block design. Observations and measurements were made 12 weeks after planting. Highly proliferating cultures were sub-cultured for further multiplication. Subculturing was performed every 6–8 weeks, 2–3 times in total until enough inoculum was obtained for the culture proliferation experiment. The culture medium used the same as the induction medium, supplemented with 2,4-D at 1.0 mg.L^{-1}. Cultures with good proliferation were selected during subculture, specifically those exhibiting a granular-nodular morphology or a mixture of granular-nodular and unorganized callus.

Morphogenic responses included the percentage of explants that grew to form buds or callus-like structures, or that grew without growth or died. Newly grown structures were categorized based on their morphologies, such as granular, nodular, and shapeless/spongy. The differentiation state of an explant was determined based on the most dominant structure that developed from one explant or inoculum. Morphological observations were conducted using a Nikon SMZ1000 stereo microscope.

Proliferation of Embryogenic Cultures

During the embryogenic inoculum proliferation stage, several experiments were conducted to optimize the culture medium, focusing on two factors:

a) A combination of auxins was tested to determine its effectiveness in promoting proliferation. A 2,4-D concentration of 1 mg.L^{-1} was compared to the combination of 1 mg.L^{-1} for each NAA and picloram and the combination of it with both 1 mg.L^{-1} NAA and 1 mg.L^{-1} IAA.

b) Nitrogen content of macro salts: The macro salts based on MS formulation were modified by altering total nitrogen and NO_3^- to NH_4^+ ratio. This included a control treatment with 60 mM total nitrogen and 30 mM total nitrogen. Additionally, adjustments were made to the NO_3^- and NH_4^+ ratios (approximately 2:1 for standard MS and 3:1 for the modification, as detailed in Table 1). For this experiment, each of the treatment media was enriched with 1 mg.L^{-1} 2,4-D and 1 mg.L^{-1} picloram

The inoculum for both experiments originated from the previous induction experiment, i.e., the friable, whitish-yellow, nodular-granular structures. A randomized block design was employed, with each treatment replicated 8 to 10 times. Each Petri dish contained 9 to 12 clumps of culture. Morphogenic responses were assessed, including the percentage of whitish friable granular-nodular structures, yellowish friable granular-nodular structures, compact nodular structures, and unorganized callus.

Somatic Embryo Development

The first experiment aimed to confirm the cultures' embryogenic nature by assessing their ability to form plantlets. Somatic embryo development was

Table 1. Inorganic nitrogen composition of MS and modified MS formulation according to treatments of total and ratios of ionic N.

Media	Total N (mM)	Ion N		Concentration in mM (g.L^{-1})	
		NO_3^-	NH_4^+	KNO_3	NH_4NO_3
Standard MS	60	41.2	18.8	18.8 (1.90)	20.61 (1.65)
½ MS	30	20.6	9.4	9.4 (0.95)	10.31 (0.83)
60 MS 3:1	60	45.0	15.0	30.0 (3.03)	15.00 (1.20)
30 MS 3:1	30	22.5	7.5	15.0 (1.52)	7.50 (0.60)

Notes: The standard MS medium contains 1.90 g.L^{-1} KNO3, 1.65 g.L^{-1} NH_4NO_3 440 mg.L^{-1}, $CaCl_2.2H_2O$, 170 mg.L^{-1} KH_2PO_4, and 370 mg.L^{-1} $MgSO_4.7H_2O$. The ½ MS consisted of a 50% dose of MS. The MS 3:1 medium is a modified MS medium with the nitrate-to-ammonium ratio adjusted to 3:1 by increasing KNO_3 and decreasing NH_4NO_3. 60 MS 3:1 consists of 3.03 g.L^{-1} KNO_3, 1.20 g.L^{-1} NH_4NO_3, 440 mg.L^{-1} $CaCl_2.2H_2O$, 170 mg.L^{-1} KH_2PO_4, and 370 mg.L^{-1} $MgSO_4.7H_2O$. 30 MS 3:1 consists of 1.52 g.L^{-1} KNO_3, 0.60 g.L^{-1} NH_4NO_3, 220 mg.L^{-1} $CaCl_2.2H_2O$, 85 mg.L^{-1} KH_2PO_4, and 185 mg.L^{-1} $MgSO_4.7H_2O$.

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investigated by varying sucrose concentrations (30 and 45 g.L^{-1}) and adjusting medium firmness by adding Gelrite at 2, 3, or 4 g.L^{-1} to the basal medium. The experiment was arranged in a randomized complete block design with four replicates, each containing twelve clumps of somatic embryo (approximately 0.7 ± 0.1 cm diameter) per Petri dish. The inoculum for the embryo development experiment comprised nodular and granular structures isolated 28 days after subculture. The cultures were placed in the dark for 2 weeks and then transferred to a room with light ranging from 4.88 to 6.10 μmol.m^{-2} per sec for a 16-hour photoperiod maintained in the same culture room as the previous experiment.

To confirm their embryogenic potential, the second experiment investigated the effect of different culture structures on somatic embryo development. Ten replicates of each treatment, consisting of nine inoculum clumps measuring 0.7 ± 0.1 cm in diameter, were plated in Petri dishes containing the treatment media. The experiment employed a randomized complete block design with two factors: morphology of inoculum and benzyl adenine (BA) concentration. These structures included: (1) friable granular or nodular structures, (2) compact, dome-shaped structures, and (3) friable unorganized callus. Each structure was cultured on a basal medium supplemented with benzyl adenine (BA) at varying concentrations (0.0, 0.5, 1.0, and 2.0 mg.L^{-1}). The treatment media were gelled with 3 g.L^{-1} Gelrite. Cultures were maintained in the dark for two weeks, followed by exposure to light with an intensity of 9.76 – 12.20 μmol.m^{-2} per sec for 16 hours within the same culture room used in the previous experiment.

Plant Conversion

Two types of SEs were used to assess the influence of somatic embryo (SE) characteristics on plant conversion: opaque and transparent/hyper-hydric. The plant conversion media consisted of the basal medium formulation used for somatic embryo development, supplemented with 30 g.L^{-1} sucrose, 3.0 g.L^{-1} Gelrite, and devoid of auxin. To evaluate the effects of gibberellin, GA_3 was added at concentrations of 0.0, 0.25, 0.5, 1.0, and 2.0 mg.L^{-1}. Fifteen SEs were plated per Petri dish, which served as a replicate. The experiment employed a randomized complete block design with ten replicates.

Data Analysis

Data was presented as the average percentage of responses from replicates of each treatment. The percentage value for each replication was derived from the percentage response value of 9 to 15 explants or inoculum-derived clumps in each petri dish. Percent germination of somatic embryos was calculated as the proportion of the number of shoots formed on one petri dish to the total number of developed somatic embryos inoculated on each petri dish.

Data was analyzed using ANOVA ($\alpha = 0.05$). To normalize the data, the percentage data were transformed with arcsine square root transformation ($x' = \sqrt{(x + 0.5)}$). Significant differences ($P < 0.05$) were further analyzed with Duncan's Multiple Range Test (DMRT) or a t-test (Student's t-test) for treatments with two levels to differentiate between treatments. For factorial experiments, when there is no interaction, the data were pooled for each factor for analysis.

Results

Induction of Embryogenic Cultures

The maturity level of zygotic embryo explants significantly affects their subsequent morphogenesis in the induction medium after 12 weeks of culture. Immature embryos developed nodular-granular and friable callus at a higher frequency (82%) compared to mature embryos (27%) (Table 2). Explants of mature zygotic embryos mostly did not grow (71%), germinated, and formed shoots in a small portion (1.57 ± 2.86%) despite the presence of 2,4-D, a strong auxin in the medium (Table 2).

Table 2. The effect of maturity of zygotic embryo explants on their morphogenic response in an induction medium containing 1.0 mg.L^{-1} 2,4-D after 12 weeks of culture

Zygotic embryo maturity	Morphogenic responses (%)		
	No growth	Shoot	Callus-nodular
Immature	17.63 ± 2.97^{b}	0.00 ± 0.00	82.37 ± 2.967^{a}
Mature	71.23 ± 4.31^{a}	1.57 ± 0.83	27.20 ± 4.385^{b}
Pr>F	<.0001**	0.070^{ns}	<.0001**

Notes: Values represent averages ± standard errors. Values in the same column followed by different letters are significantly different, according to the t-test.

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The concentration of 2,4-D and picloram significantly affected the morphogenic responses of immature zygotic embryo explants. When exposed to a medium containing either 2,4-D or picloram, the explants might have sprouted shoots prematurely (precocious germination), formed callus, a mass of undifferentiated cells, in two variations: soft, watery, and translucent, or compact and yellowish, developed granular-nodular structures, which appeared round and resembled embryos, shown no growth and died. Increasing the concentration of 2,4-D 0.25 to 5.00 mg.L^{-1} significantly increased the nodular and callus formation percentage but then decreased at 10 mg.L^{-1} (Figure 1A). However, low concentrations of 2,4-D (< 0.25 mg.L^{-1}) have a significant effect on shoot development (around 5 to 15%). Increasing 2,4-D concentration progressively decreased shoot development and inhibited it at 1 mg.L^{-1} (Figure 1A). Like 2,4-D, increasing picloram concentration increased the percentage of explants forming callus and nodular-globular structures. However, the maximum percentage was at a concentration of 5 mg.L^{-1}, with an insignificant decrease observed at 10 mg.L^{-1} (Figure 1B). The effect of picloram concentration on shoot development mirrored that of 2,4-D. However, complete inhibition occurred at a slightly higher concentration of 2.5 mg.L^{-1} (Figure 1B).

Proliferation of Embryogenic Cultures

The morphology of proliferating cultures of wild Musa can be categorized into four main structures (Figure 2): a) whitish, friable nodular granular structure; b) yellowish, friable nodular structure; c) whitish, compact nodular structure; d) Friable, cottony-irregular callus. The experiment comparing auxin composition in the proliferation medium revealed that media containing 2,4-D did not affect inoculum morphology compared to those containing NAA, IAA, and picloram. As shown in Table 3, the medium containing only 1 mg.L^{-1} 2,4-D tended to produce a higher percentage of callus with a cotton-like structure than other media compositions. Adding NAA, IAA, and picloram decreased the formation of irregular callus and increased the presence of the organized nodular-granular structure, resembling globular embryo-like structures.

The modified macro-MS experiment, investigating the effects of total nitrogen concentration and the NO_3^- to NH_4^+ ratio, did not reveal significant impacts on inoculum structure and morphology. Half-strength macro-nutrient media can maintain inoculum proliferation and increase the percentage of embryogenic aggregates (friable nodular-granular) while decreasing those with friable-irregular morphology (Table 4).

Somatic Embryo Development and Plant Conversion

The development of the somatic embryo was affected by the interaction of sucrose and Gelrite concentration in the medium (Pr>F= 0.0215). The combination of 30 g.L^{-1} sucrose and 3.0 g.L^{-1} Gelrite resulted in the highest somatic embryo development score, 3.4, representing about 60% of culture-forming somatic embryos (Table 5). A higher concentration of both, i.e., 45 g.L^{-1} sucrose and 4.0 g.L^{-1} Gelrite, also resulted in similar scores (Table 5).

The use of various concentrations of BA for development and subsequent plant conversion from different types of inocula showed no interaction between those two factors (Pr>F =0.1759). The

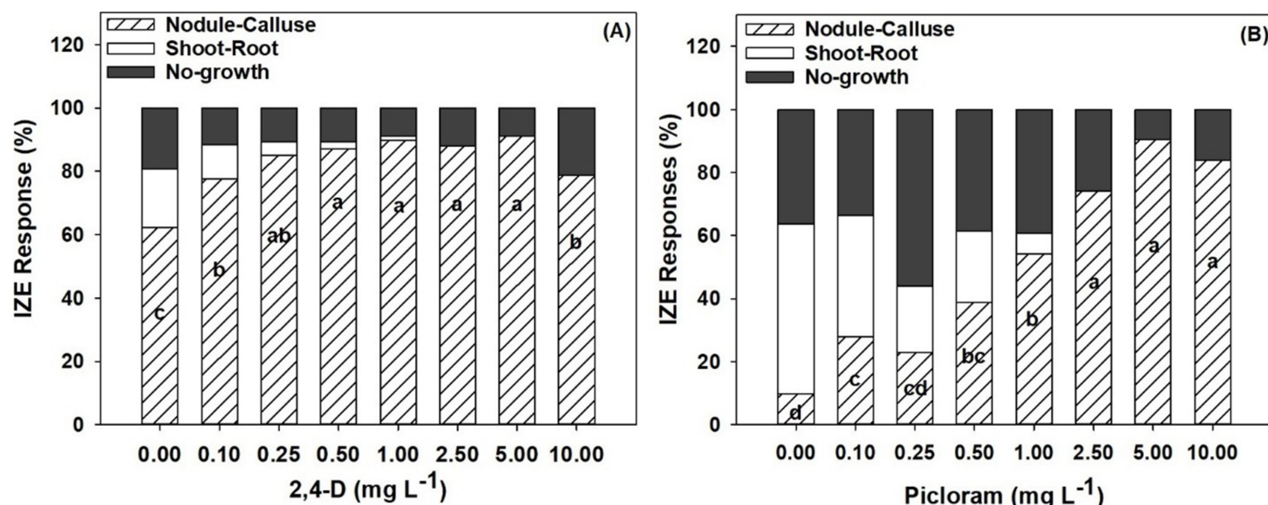

Figure 1. Immature zygotic embryos explants response to 2,4-D (A) and picloram (B) at concentration 0 – 10 mg.L^{-1}. Values represent the percentage of the explant response. Different letters in the same category morphology are significantly different according to the Duncan multiple range test at α=0.05.

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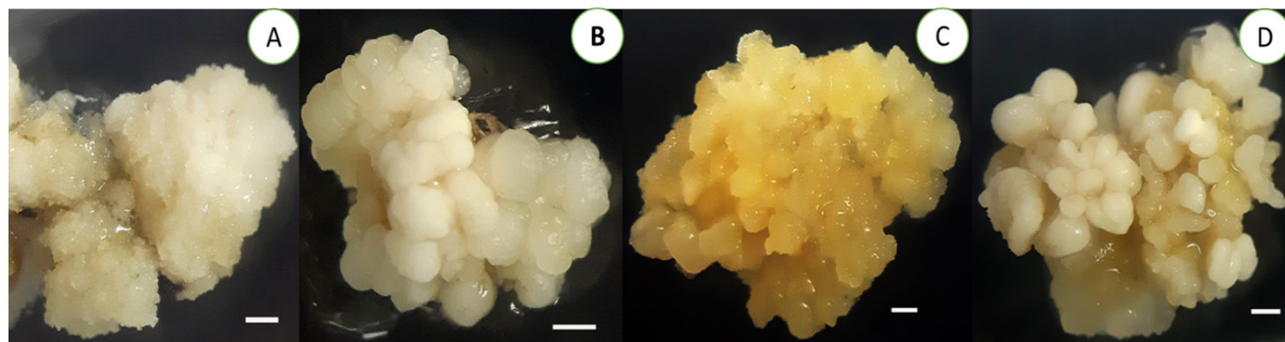

Figure 2. Representative morphology of embryogenic culture from subspecies *malaccensis* on proliferation medium; A) irregular-spongy callus; B) compact nodular; C) Friable, yellowish nodular-granular; D) Friable, whitish nodular-granular. Scale bar = 1 mm.

Table 3. The effect of 2,4-D and its combination with other auxins on the morphologies of the proliferating cultures

Auxins (mg.L^{-1})	Culture morphology (%)			
	Friable granular $^{x)}$	Friable nodular $^{y)}$	Compact nodular $^{x)}$	Friable cottony $^{x)}$
2,4D (1)	11.82 ± 4.59	46.77 ± 2.65	22.82 ± 3.66	18.59 ± 2.57
2,4D (1) + NAA (1)	6.61 ± 4.73	47.77 ± 4.64	34.52 ± 7.30	11.11 ± 2.63
2,4D (1) + Pic (1)	5.40 ± 2.26	51.73 ± 5.30	30.56 ± 5.40	12.31 ± 3.80
2,4D (1) + NAA (1) + IAA (1)	12.19 ± 5.93	42.28 ± 3.73	31.44 ± 5.25	14.08 ± 2.42
Pr>f	0.0570^{ns}	0.6828^{ns}	0.3232^{ns}	0.0768^{ns}

Notes: Values represent averages ± standard errors. Values in the same column followed by different letters are significantly different according to Duncan's multiple range test at α= 5%. $^{x)}$ Friable and compact nodular and friable cottony cultures are white. $^{y)}$ friable nodular cultures are yellow.

Table 4. The effect of nitrogen concentrations and macro salts' compositions on the proliferating cultures' predominance morphologies

Medium	Culture morphology (%)			
	Friable granular$^{x)}$	Friable nodular $^{y)}$	Compact nodular$^{x)}$	Friable cottony$^{x)}$
MS standard	3.14 ± 1.08	57.73 ± 4.06	16.32 ± 2.79	22.80 ± 4.72
½ MS	0.52 ± 0.30	62.26 ± 3.34	21.74 ± 2.42	15.48 ± 3.18
60MS 3:1	3.37 ± 1.78	54.65 ± 5.04	21.01 ± 2.97	20.97 ± 5.25
30MS 3:1	3.47 ± 2.91	58.82 ± 4.78	22.67 ± 2.80	15.03 ± 3.95
Pr>f	0.2516^{ns}	0.4412^{ns}	0.3751^{ns}	0.3855^{ns}

Notes: Values represent averages ± standard errors. Values in the same column followed by different letters are significantly different according to Duncan's multiple range test at α= 5%. $^{x)}$ Friable and compact nodular and friable cottony cultures are white. $^{y)}$ friable nodular cultures are yellow.

Table 5. The effect of sucrose and Gelrite concentrations on the somatic embryo development scores

Sucrose (g.L^{-1})	Gelrite (g.L^{-1})			Mean
	2.0	3.0	4.0	
30	1.7 b	3.4 a	2.8 a	2.6
45	2.8 a	2.5 ab	3.5 a	2.9
Mean	2.2 y	3.0 x	3.1 x	

Notes: SE development score represents the percentage range of inoculum that formed somatic embryos. Score 1: <20%; score 2: 21 – 40%; score 3: 41 – 60%, score 4: 61 – 80%; score 5: > 80%; the same letter indicates no significant difference in response based on Duncan multiple range test at α= 5%. Data represent the average score of 12 clumps from six replicates.

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friable granular structures composed of proembryos produced a significantly higher number of somatic embryos, followed by the compact structures that were essentially somatic embryo clumps, while the friable irregular callus produced virtually no somatic embryos (Table 6). The somatic embryos could sprout, forming shoots, and some of the shoots formed roots in smaller numbers (Table 6). The conversion rate from friable granular-nodular inoculum was calculated to be 11.6%, while the conversion rate for compact inoculum was 5.91%. Adding BA at any concentration significantly decreases the number of somatic embryos developed from each clump, but 2 mg.L^{-1} BA substantially increases the number of somatic embryos that sprout, form shoot, and root (Table 6). The low plant conversion rate was likely due to insufficient observation time, making most embryos immature. Normal somatic embryos could regenerate and differentiate to form roots and shoots. In contrast, some somatic embryos converted into abnormal shoots without roots, while others only formed roots (Figure 3). This low conversion rate could result from precocious germination.

The opaque somatic embryos displayed significantly higher sprouting rates than hyper-hydric ones (Table 7). On the contrary, the hyper-hydric embryos had a significantly higher number of embryos that failed to form shoots (Table 7). Hyper-hydric embryos also produced hyper-hydric shoots that generally were unable to acclimatize. Overall, opaque somatic embryos resulted in better plant conversion with higher sprouting and more normal shoots than hyper-hydric SEs (Table 7).

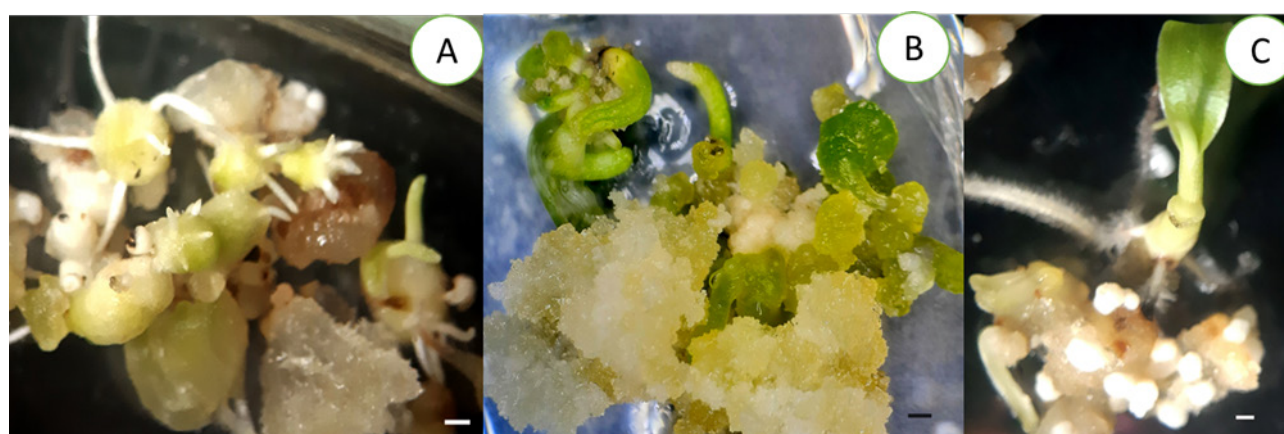

Figure 3. Regeneration of somatic embryo of wild Musa on plant conversion medium; A) roots only; B) abnormal shoots, without roots; C) a normal shoot.

Table 6. The effect of inoculum morphology and BAP concentrations on the development of somatic embryos and their subsequent plant conversion eight weeks after inoculation

	Treatment	∑ Opaque embryo	∑ sprouting somatic embryo	∑ shoots	∑ roots
Morphology inoculum	Friable nodular-granular	11.44 a	2.35 a	1.90 a	0.29 a
	Compact nodular	4.91 b	0.45 b	0.29 b	0.09 a
	Friable-cottony	0.56 c	0.30 b	0.11 b	0.00 a
	Pr>f	<.0001**	0.0008**	0.0482*	0.7417^{ns}
BAP (mg.L^{-1})	0.00	8.14 a	0.50 b	0.53 b	0.19 a
	0.50	6.42 a	0.84 ab	0.77 ab	0.16 a
	1.00	5.52 a	1.28 ab	1.04 ab	0.00 a
	2.00	5.62 a	2.42 a	1.52 a	0.26 a
	Pr>f	0.1053^{ns}	0.0367*	0.0387*	0.5313^{ns}

Notes: The data represents the average number of responses per clump inoculum with a 0.7 ± 0.1 cm diameter. There are nine inoculum clumps per Petri dish. Similar letters in the same column and treatment indicate no significant differences based on Duncan's multiple range test at α= 5%. There is no interaction between callus morphology and BAP concentration on the response, so the data is pooled for each factor.

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Supplementation with GA_3 significantly improved plant conversion, as indicated by variables of somatic embryo sprouting or germination (Table 7). Somatic embryo sprouting increased with increasing GA3 concentration, reaching a maximum of 2.00 $mg.L^{-1}$. Higher GA_3 concentrations tended to decrease the percentage of embryos failing to convert. While increasing GA_3 concentration improved sprouting and decreased non-growth SEs, with 0.25 – 0.50 $mg.L^{-1}$ showing the best results, it did not significantly affect other parameters. GA_3 presence in the medium did not affect the development of normal or hyper-hydric shoots (Table 7). The high proportion of hyper-hydric shoots developed from somatic embryos suggests that further optimization of the plant conversion protocol is necessary.

We hereby summarize the different medium formulations employed for each of the stages of somatic embryogenesis of wild banana *M. acuminata* ssp. malaccensis, from induction to proliferation, somatic embryo development, and plant conversion (Table 8).

Discussion

This study reports an efficient and complete protocol for somatic embryogenesis and regeneration in the wild banana subspecies Musa *acuminata* ssp. *malaccensis*. The protocol utilizes semi-solid medium and immature zygotic explants. We optimized the entire process, encompassing induction, proliferation, embryo development, and plant conversion, and documented the morphological changes throughout.

Using immature zygotic embryos as explants significantly improved the efficiency of initiating embryogenic cultures. Immature embryos can produce more somatic than mature embryos when cultured in vitro. A similar result was reported by Uma et al. (2012) that the regeneration rate of embryogenic calli into plantlets (76.6%) was higher from immature zygotic explants than from mature embryos (50.6%). This difference may be because young tissues during seed development have cells that differentiate early and rapidly. Additionally, the level of endogenous hormones may also influence the regeneration response. In contrast, cultures derived from mature embryos often have a poor regeneration response and fail to grow embryogenic culture.

The success of embryogenic culture induction might be attributed to the rich culture medium used. This medium was formulated by selecting components from various successful protocols of somatic embryogenesis in banana development by Strosse et al. (2003) and Escobedo-Gracia Medrano (2016), including the standard MS macro and micronutrients, myoinositol, White's vitamins and organics (as a complete formulation published by Murashige and Skoog, 1962), with additional components like Biotin (Khalil et al., 2002), PVP (Roostika et al., 2022), L-glutamine (Husni et al., 2014), casein hydrolysate and proline (Khalil et al., 2002; Smitha et al., 2020). Furthermore, successful culture establishment was influenced by the selection method for inoculum during subsequent subcultures. This method, termed selective subculture, involved choosing friable granular structures for further propagation.

Table 7. Plant conversion from somatic embryos as affected by somatic embryo morphology and GA_3 concentration in the medium eight weeks after culture

Treatment		Sprouting (%)	Shoots (%)	Rooted (%)	Non-growth (%)	Shoot (%)	
						Normal	hyper-hydric
Somatic embryo	Opaque	47.94 a	13.33	22.86	23.49 b	90.78 a	9.22 b
	Transparent	27.47 b	12.80	19.47	51.20 a	23.15 b	76.85 a
	Pr>f	<.0001**	0.5002^{ns}	0.0823^{ns}	0.0003**	<.0001**	<.0001**
GA_3 ($mg.L^{-1}$)	0.00	26.67 b	16.30	6.67	50.37 a	53.99	46.01
	0.25	32.50 ab	17.50	18.33	31.66 a	64.96	35.04
	0.50	41.48 a	10.37	6.67	41.48 a	54.58	45.42
	1.00	36.30 ab	11.11	19.26	33.33 ab	46.05	53.95
	2.00	45.93 a	13.33	19.26	21.48 b	59.16	40.84
	Pr>f	0.0438*	0.8271^{ns}	0.0823^{ns}	0.0098*	0.1208^{ns}	0.2259^{ns}

Notes: Data represents the average percentage of somatic embryo formation (15 somatic embryos per Petri dish). Means within the same column and treatment with similar letters are not significantly different (α = 0.05, DMRT test). There was no interaction between somatic embryo morphology and GA_3 concentration on the somatic embryo formation, so the data were pooled for each factor. The medium was supplemented with 0.5 mg L^{-1} BAP.

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The type of growth regulator in the medium also played a role, with picloram being more effective at higher concentrations than 2,4-D. The synthetic auxin-like compound 2,4-D is a major inducer of somatic embryogenesis, affecting the expression of embryogenesis-related genes through a complex network of auxin signaling pathways (Wójcik et al., 2020). Supplementing the medium with other auxins besides 2,4-D, such as picloram, NAA, or IAA, can further enhance the chances of explant development into embryos. Combinations of auxins can promote cell division and proliferation, and different auxin types can elicit distinct physiological responses in plant materials during somatic embryogenesis (Hazubska-Przybył et al., 2020). Somatic embryogenesis in bananas from male flower buds can be induced with 4 mg.L^{-1} 2,4-D, 1 mg.L^{-1} IAA, and 1 mg.L^{-1} NAA (Kumaravel et al., 2020; Natarajan et al., 2020).

In this study, the macro base medium composition did not significantly affect culture morphology at the proliferation stage, including the effects of total nitrogen or the nitrate/ammonium ratio. The medium composition and total nitrogen content used in the experiment might already provide an optimal range for embryo proliferation. Nitrogen levels may play a secondary or supporting role that becomes less apparent when PGRs are optimized. High concentrations of ammonium as a nitrogen source can hinder somatic embryo differentiation and increase hyper-hydricity. In contrast, a higher proportion of nitrate can improve somatic embryo quality and reduce hyper-hydricity (Polivanova and Bedarev, 2022). Half-strength MS medium has been successfully employed for banana plant embryogenesis in various applications, including SEs suspension culture (Rustagi et al., 2019) and SEs maturation (Ponni and Nair, 2019; Natarajan et al., 2020). Meanwhile, half the strength of the macro-MS medium produced the highest embryogenesis rate in *Paris polyphylla* Sm. plants from immature embryo explants (Raomai et al., 2014), the optimal medium strength can vary depending on the plant species.

The frequency of successful somatic embryos (SEs) development for subspecies *malaccensis* could be improved by selecting granular pro-embryonic masses as inoculum and increasing the concentration of gelling

Table 8. The composition of media employed for somatic embryogenesis of wild banana *M. acuminata* ssp. *malaccensis* of for culture induction and proliferation, somatic embryo development, and subsequent plant regeneration

Medium component	Induction medium	Proliferation medium	Somatic embryo development medium	Plant conversion medium
Modified MS* macro salts				
NH_4NO_3 (g.L^{-1})	1.200	0.600	1.200	1.200
KNO_3 (g.L^{-1})	3.030	1.520	3.030	3.030
$CaCl_2$ (g.L^{-1})	0.440	0.220	0.440	0.440
$MgCl_2$	0.170	0.085	0.170	0.170
KH_2PO_4	0.370	0.185	0.370	0.370
Micro salts	MS	MS	MS	MS
FeNaEDTA (mg.L^{-1})	36.70	36.70	36.70	36.70
Vitamin	MS	MS	MS	MS
Glycine (mg.L^{-1})	2.0	2.0	2.0	2.0
Biotin (mg.L^{-1})	1.0	1.0	1.0	1.0
Myo Inositol (mg.L^{-1})	100	100	100	100
L-Glutamine (mg.L^{-1})	100	100	100	100
Casein hydrolisate (mg.L^{-1})	250	500	250	250
Proline (g.L^{-1})	5	5	5	5
PVP (MW 360,000) (mg.L^{-1})	10	10	10	10
Hormone (mg.L^{-1})	2,4-D (1) or Picloram (5)	2,4 D (1) + Picloram (1)	-	BA (0.5) + GA_3 (0.5)
Sucrose (g.L^{-1})	30	30	30 - 45	30
Gelrite (g.L^{-1})	2.0	2.0	3.0 - 4.0	3.0

Note: * Murashige and Skoog (1962) medium.

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agent or sucrose in the medium. Proembryos, due to their integrative development, can spontaneously form somatic embryos. Increasing the sucrose and gelling agent concentration in the medium has improved somatic embryo development in avocados (Witjaksono and Litz, 1999) and oil palm (Palanyandy et al., 2020). A higher gelling agent concentration restricts water availability in the medium (Muzika et al., 2024); this prevents hyper-hydricity.

Benzyl adenine (BA) promotes somatic embryo development and regeneration. While BA can increase the germination of somatic embryos, high levels may decrease their development. The optimal BA concentration and desired outcome must be determined for each plant species. A study by Morais-Lino et al. (2016) showed a 40% germination rate for banana somatic embryos with 0.8 µM BA. Khalil et al. (2002) employed a two-stage culture approach with BA, using a higher concentration (5 mg.L^{-1}) for initial embryo differentiation followed by a lower concentration (1 mg.L^{-1}) for plantlet development. However, high BA levels can inhibit somatic embryo development by rapidly promoting cell division (Asghar et al., 2023), potentially disrupting normal embryo development and triggering premature germination (Mendez-Hernandez et al., 2019).

Plant conversion, defined as the development of shoots and complete plantlets, is followed by further histodifferentiation of mature somatic embryos for the scutellum and sprouting of the apical meristems. Sprouting and shoot formation are more efficient from opaque, non-hyper-hydric somatic embryos. Hyperhydricity is a particular challenge for somatic embryos of highly embryogenic genotypes. These genotypes rapidly produce cotyledonary stage embryos following subculture from a 2,4-D-containing medium. However, these embryos often become hyper-hydric during the early cotyledonary stage, exhibiting translucent, water-soaked, discolored, and brittle characteristics. Consequently, they cannot fully mature and have poor germination rates. Increasing agar (solidifying agent) concentration in the culture medium can reduce hyper hydricity in somatic embryos (Polivanova and Bedarev, 2022), as observed in shallot somatic embryogenesis (Handayani and Witjaksono, 2023).

Adding GA_3 to the medium can increase the germination of somatic embryos (Paiva et al., 2023), but it can also negatively impact their subsequent development. While higher GA_3 concentrations decrease the percentage of embryos failing to convert, these studies have shown that adding low concentrations (0.25 - 0.50 mg.L^{-1}) of GA_3 can increase somatic embryo germination without compromising embryo conversion rates into plants. Furthermore, increasing plant conversion can be achieved by adding GA_3 alone or in combination with cytokinin/ auxin. For example, Tripathi et al. (2022) successfully used 1.0 mg.L^{-1} GA_3 combination with 2.0 mg.L^{-1} TDZ to increase plant conversion in sandalwood. Higher GA_3 concentrations also decreased the percentage of embryos failing to convert. Unfortunately, the low regeneration rate achieved from somatic embryos still requires further optimization of the plant conversion protocol.

This technique has the potential to significantly increase somatic embryogenesis efficiency and become a valuable tool for expanding the use and utilization of wild bananas in pre-breeding activities to improve banana genetics in the future. Further studies are necessary to optimize the plant conversion protocol.

Conclusions

This study established an efficient and complete protocol for inducing somatic embryogenesis and regeneration in the wild banana subspecies *Musa acuminata* ssp. *malaccensis*. The protocol utilizes immature zygotic embryos cultured on a semi-solid medium. Immature zygotic embryos were significantly more efficient at initiating embryogenic cultures than mature embryos. Induction was achieved on a complex modification of MS medium supplemented with 1 mg.L^{-1} 2,4-D auxin or 5 mg.L^{-1} picloram. The proliferation rate of embryogenic cultures was highest used combination of 2,4-D and picloram, each 1 mg.L^{-1}. A half-strength basal medium was sufficient to sustain proliferation until embryo formation and regeneration of the cultures into shoots. The frequency of shoot formation from the developed somatic embryos was improved by incorporating 0.5 mg.L^{-1} BA and 0.5 mg.L^{-1} GA_3 into the regeneration medium.

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