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The effect of auxins and cytokinin combinations on leaf culture in edible and ornamental banana cultivars

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Abstract

Banana is a fruit crop with economic importance. This research focuses on banana cultivation, aiming to develop new methods and establish protocols for micro-propagation using leaf culture, supporting future breeding efforts. The study utilized half-strength MS medium with varying auxin and cytokinin concentrations to induce callus formation under sterile conditions. Callus formation rates were assessed across treatments, and successful Calli were transferred to proliferation and induction media for shoot and root induction. The results showed that specific concentrations of auxin and cytokinin combination significantly influenced callus induction in Dwarf banana leaves. The highest callus formation occurred with 4 mg/L 2,4-D + 0.5 mg/L NAA, while 0.5 mg/L TDZ + 0.5 mg/L NAA was most effective for callus proliferation. Root induction succeeded with 0.5 mg/L TDZ and 1 mg/L IBA, in contrast, shoot regeneration was unsuccessful. Additionally, the study examined anti-browning and antioxidant properties, revealing that no white or yellow callus developed under any of the treatments. Although this research successfully achieved callus induction, shoot regeneration from banana leaf callus remains a challenge. Due to the complexity of the process and the innovative techniques involved, further studies are needed to explore this stage, especially considering the time constraints of the current research.

Keywords: Auxins; Cytokinin; Combinations; Micropropagation; Cultivars

1. Introduction

Banana (Musa spp.) is a type of perennial herbaceous monocot plant. Bananas and plantains, which belong to the Musaceae family and are scientifically referred to as Musa spp., play a crucial role as staple crops worldwide. These fruits originated in Malaysia, evolving through a complex hybridization process (Novak, 1992; MD. AL-AMIN1, 2009). Banana has three genera, Musa, Musella and Ensete. 235 scientific plant names of species rank for the family Musaceae. Of these 78 are accepted species names (The plant list, 2023). The banana is a highly significant tropical fruit that holds great importance as a cultivated crop. Global production of bananas is estimated to be around 113.9 million metric tons (FAO, 2017) reaching approximately 135 to 140 million tons annually by 2022–2024. The banana is a fruit crop that originates from tropical regions and is cultivated in more than 122 countries across the globe. Prior to 2004, it held the fourth position in terms of agricultural land usage, with a cultivated area spanning 3.8 million hectares. Additionally, the total production of bananas amounted to 56.4 million metric tons. This ranking placed bananas behind rice, corn, and milk in terms of overall production (Chai et al., 2004; Arumugam and Manikandan, 2011; Gajendra Kumar Rana, et al., 2018). Southeast Asia is recognized as the primary center for the origin and diversity of bananas (Musa spp.). This region harbors a rich abundance of banana varieties, including both wild species and cultivated ones (Hapsari, L., et al (2015); Espino et al., 1992; Nasution, 1991).

Banana is generally propagated vegetatively through suckers, this process is very slow as the rate of multiplication of suckers through conventional vegetative means has been found to express several negative impacts which include

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transmission of diseases, low production, and poor preservation of original plant genetic material (Hussein, 2012). Micropropagation will be another option to increase the quantity and quality of production. Micropropagation refers to the process of cultivating plants in a controlled laboratory environment, allowing them to regenerate from cells, protoplasts, tissues, or organs. This regeneration occurs on specialized nutrient media. Given suitable conditions, a complete plant can develop from just a single cell. This technique enables the generation of numerous identical copies of a particular plant using methods of tissue culture (Ortiz, R., and Vuylsteke, D. 1996, October). Banana tissue culture is one of the techniques that is very useful, large quantities of banana plantlets are produced within a short period and keep the original plants (Selvakumar, S., and Parasurama, D. S. 2020). Banana plantlets generated using micropropagation techniques exhibit quicker establishment, enhanced robustness, a shorter growth cycle, and greater yield compared to those produced using traditional methods (Kumar, A., Kumari, P., and Shukla, L. N. 2012). In plant tissue culture, browning is a major challenge in banana tissue culture, causing high mortality in mass micropropagation of Cavendish bananas due to lethal browning of plantlets (Ko, W. H., Su, C. C., Chen, C. L., and Chao, C. P. 2009). Grasping nitrogen assimilation and its roles in plant growth is essential for understanding cell differentiation in plants. The type and amount of nitrogen in the in vitro medium significantly impact cell growth, differentiation, and totipotency (Sebasigari, K., Persley, G. J., and De Langhe, E. A. 1987). Reducing nitrogen application often initiates sexual development (Trewavas, A. J. 1983). Sucrose, the most used carbon source, promotes growth and development at all stages of micropropagation.

The types of PGRs and their formulations are known to have a species and cultivar-specific impact on the in vitro re-generation of bananas from shoot tip explants (Bairu, M. W., Stirk, W. A., Doležal, K., and Van Staden, J. 2008). This research will focus on different varieties of edible bananas that hold potential for commercial production and typically grown in banana gardens and plantations. There are many research articles reporting banana tissue culture by sucker, whereas the propagation by leaves has not been achieved. Even though, banana shoot-tip propagation shows high propagation efficiency, the plant will be lost in this method. Furthermore, leaf tissue culture will enable to minimize the damage on rare and expensive banana species. It is expected that the leaf tissue culture could be alternative method of banana propagation.

There are many factors affecting micropropagation of fruit crops (Hassan, S. A. M., and Zayed, N. S. 2018). However, in this study we will carries on some factors which included the type of explants used for micropropagation, establishment of callus induction from leaf explant and the shoot and root induction methods. Establishing contamination-free plantlets is a vital step to eliminate pest and pathogen pressure during the production cycle and promote callus growth. The appropriate culture media composition, containing auxin and cytokinin tailored to each banana plant species, is essential for inducing callus, shoot, and root development from leaf tissue. The purpose of this research to elucidate the ideal composition of culture media that can stimulate the development of callus, shoots, and roots from leaf tissue in both common and rare banana cultivars and to achieve the generation of new plants from banana leaf tissue using callus culture techniques.

2. Materials and methods

2.1. Plant materials

The research was conducted in the Laboratory of Horticultural Science, Faculty of Agriculture, Kyushu University. The plant material used edible banana: Musa acuminata cv. Dwarf banana (Sansyaku Banana), Musa luikuiensis (Okinawa Shima Banana), Musa paradisiaca cv. Latundan (Apple Bananas 1), Musa spp. cv. 'cavendish' (Mansuke banana); Musa paradisiaca cv. Ice cream (Ice cream banana); Musa acuminata x balbisiana (AAB Group) (Apple banana 2); Ornamental banana: Musa x paradisiaca 'Ae Ae'.

2.2. Establishment of leave callus induction

2.2.1. Medium and explant preparation

Culture medium preparation

Culture medium for this experiment was the basal medium (macro, micronutrients, and vitamin) of Murashige and Skoog, (1962) (Dalton, C. C., Iqbal, K., and Turner, D. A. 1983). A half strength macro-elements MS media was applied with different plant growth regulators (PGRs) and the pH was adjusted to 5.6. before autoclaving. PGRs (Cytokinin and Auxin) were modified from the best growth of shoot callus induction in banana (Kumar, R., Ahmed, M. F., Mir, H., Mehta, S., and Sohane, R. K. (2019). The medium was put in autoclave in 110 °C in 1 minute and filled in the 120 ml flasks, then completed by 121 °C in 15 minutes.

Medium of callus induction

Various combination of auxin and cytokinin with their concentration for callus induction (Table 1.)

Surface sterilization

The disinfection procedure is initiated from young leave of banana had been cut into 20–25 cm, cleaned with trap and distilled water, soaked in ethanol 70% (5 min) then 2% Sodium hypochlorite (NaClO2) (10 min) rinse with distilled water from autoclaves 3 times.

2.2.2. Leave callus induction implementation

After completing the contaminant-free stage, banana leaves were cut into small pieces (1 x 1 cm) and placed into the culture medium. These procedures were conducted in a Bio Clean Bench. All cultures were incubated in a culture room at 25 °C with a relative humidity of 55 % and a 16/8 h light/dark photoperiod provided by 40 W cool white, fluorescent tubes positioned 20-50 cm above the bench surface. Data on callus formation were collected every two weeks, and the explants were monitored for 16 weeks to observe callus formation. Callus formation was observed in each explant, and the rates of callus formation were compared across different treatments.

2.2.3. Callus proliferation

After 12 weeks of in vitro callus growth, the Calli were transferred to a proliferation medium containing a combination of auxin and cytokinin at concentrations of 0.5 mg/L NAA, 1 mg/L 2,4-D, and 0.5 mg/L TDZ. All sub-cultured Calli were maintained in a culture room at 26°C with a 16/8 h light/dark photoperiod. Measurements of callus diameter were recorded on a weekly basis.

2.2.4. Shoot induction

The Calli were transferred to a shoot induction medium, which was a modified liquid ½ MS medium supplemented with the optimal formula for shoot regeneration: 3 mg/L BAP and 1 mg/L NAA (Koarapatchaikul, K., and Kanchanapoom, K. 2010). The cultures were maintained in 120 ml flasks at a temperature of 25 °C with a 16/8 h light/dark photoperiod and a light intensity of 23.2 μmol m^{-2} s^{-1}. Data on shoot formation were collected every week.

2.2.5. Root induction

The Calli were sub-cultured to a root induction medium consisting of a half strength MS medium supplemented with 0.5 mg/L TDZ and 1 mg/L IBA (Subrahmanyeswari, T., Gantait, S., Sarkar, S., and Bhattacharyya, S. (2022). The pH was maintained at 5.7. The cultures were kept at 25°C with a 16/8 h light/dark photoperiod. Data on rooting were collected in weekly.

2.3. Studies on anti-browning and antioxidant of callus induction

This study aims to reduce browning in the formation of dead or black callus. Therefore, the culture medium used activated charcoal (AC) and optimized the PVP with ascorbic acid methodology, employing a half-strength MS medium supplemented with either auxin alone or a combination of auxin and cytokinin. The activated charcoal treatment medium was a ½ MS medium containing 3-4 mg/L 2,4-D and 0.5-1 mg/L NAA with 200 mg/L activated charcoal. For optimizing anti-browning, another treatment used a ½ MS medium supplemented with 3-4 mg/L 2,4-D, 0.5-1 mg/L NAA, BAP, and TDZ, along with 2 mg/L PVP and 0.5 mg/L ascorbic acid. All cultured explants were stored in a culture room at 25 °C with a 16/8 h light/dark photoperiod. Callus formation data was collected every two weeks.

2.4. Studies on influence of nitrogen and carbon source in callus induction

This research aligns with Shanjani (2003) by utilizing a lower amount of total nitrogen in the medium. To minimize nitrogen sources, the macro elements of MS medium (Stock 1) were modified to half their concentration, specifically potassium nitrate (KNO_3) and ammonium nitrate (NH_4NO_3). Additionally, the optimization of sucrose from 3% to 2%, as suggested by Hossain et al. (2013) who studied the impact of carbon sources on callus induction and regeneration ability in banana, was followed. They found that the highest percentages of shoot and root formation were obtained with media containing 3% and 2% sucrose, respectively. However, our study used 100%, 70%, and 50% sucrose (Table 5) with a half-strength macro-element of NH_4NO_3 and KNO_3 to induce callus formation in each treatment.

2.5. Experimental design, data collection and analysis

The experiments were carried out using a completely randomized design (CRD), with each trial repeated three times and 16 explants (or plantlets) per treatment/substrate to minimize potential errors. The collected data were stored in an Excel file for subsequent analysis. Using Excel Microsoft office, the data were analysed to calculate the callus formation rate and to compare the different treatments.

3. Results

3.1. Callus induction

Among the 9 combinations of auxins and 37 combinations of auxins and cytokinin with various concentrations, four banana cultivars were tested. Result revealed that in a single banana cultivar (*M. acuminata* cv. Dwarf banana), four different media produced white/yellow callus formation. Among these, the medium containing half-strength MS supplemented with 4 mg/L 2,4-D and 0.5 mg/L NAA produced the highest rate of callus formation which responses (5.81%). While the explant obtained by 3 mg/L 2,4-D + 1 mg/L NAA induced callus (4.30%), then followed by 3 mg/L 2,4-D + 0.5 mg/L NAA and 3 mg/L 2,4-D + 1 mg/L NAA presentively (Table 1). Additionally, extensive research examined the formation of black/dead callus under various treatments (Figure 1.). However, this specific type of callus consistently did not progress into white/yellow callus. Consequently, it was determined to be nonviable and considered unsuitable for further experimental stages. Additionally, the white/yellow callus was characterized by its color, texture, and growth properties. Furthermore, three more banana cultivars were treated in the same medium as the first cultivar (*M. acuminata* cv. Dwarf banana). The results showed that no white or yellow callus was induced by any treatment. Instead, black or dead callus was promoted by *M. luikuiensis* when a half-strength MS medium containing 2,4-D and KT was used, it was reached (83%) (Table 2). Ornamental banana leaf culture total 17 different combinations of auxin and cytokinin were tested, the results indicated that none of the treatments induced the formation of white or yellow callus; instead, they all produced black or dead callus. The highest rate of black callus formation, reaching 100%, was observed with the combination of a half-strength MS medium containing either 4 mg/L 2,4-D + 1 mg/L BAP or 4 mg/L 2,4-D + 1 mg/L TDZ (Table 3). However, this type of black callus was not significant for shoot regeneration.

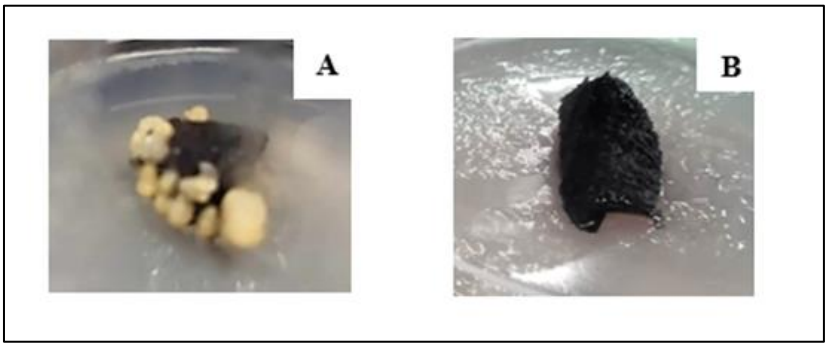


Figure 1 Callus formation (A) white/yellow Calli forming, (B) Black/dead Calli forming

However, the morphological variation of Calli was observed and categorized as follows:

- Type I: Transparent water callus. These soft Calli were white, smooth, wet, and easily broken into small pieces upon touch. They were induced on media supplemented with 3 mg/L 2,4-D + 0.5 mg/L NAA and 4 mg/L 2,4-D + 1 mg/L NAA.
- Type II: White callus with small clusters. These Calli were compact and white with nodular structures, highly competent for forming embryonic structures. This type was regenerated on media containing 4 mg/L 2,4-D + 0.5 mg/L NAA. The growth rate of these Calli was relatively slow.
- Type III: Yellow callus with large clusters. These Calli were very hard, compact, and globular with thickened walls and intercellular spaces. They developed on media with 3 mg/L 2,4-D + 1 mg/L NAA, maintained their texture and structure when sub-cultured, grew very slowly, turned yellow-brown, and died after 6 months (Figure 2).

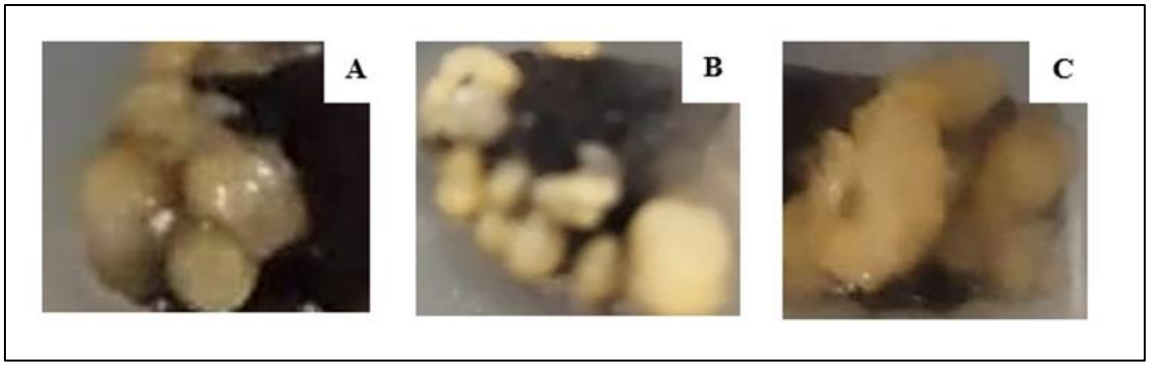


Figure 2 The morphological callus classification (A) Callus Type I: transparent water callus; (B) Callus Type II: White callus with small clusters; (C) Callus Type III: Yellow callus with large clusters

3.2. Callus proliferation

Calli were placed in the medium to observe their growth conditions. The results revealed that during the 8-week monitoring cycle, callus type II exhibited the largest growth. Specifically, in a medium supplemented with a combination of 0.5 mg/L NAA and 0.5 mg/L TDZ, the callus grew to 0.80 cm. In contrast, the medium containing three PGRs (0.5 mg/L NAA, 1 mg/L 2,4-D, and 0.5 mg/L TDZ) resulted in slower growth, with the callus reaching 0.73 cm. Notably, the treatment with 1 mg/L 2,4-D and 0.5 mg/L TDZ promoted very slow callus growth, achieving only 0.43 cm (Figures 3 and 4).

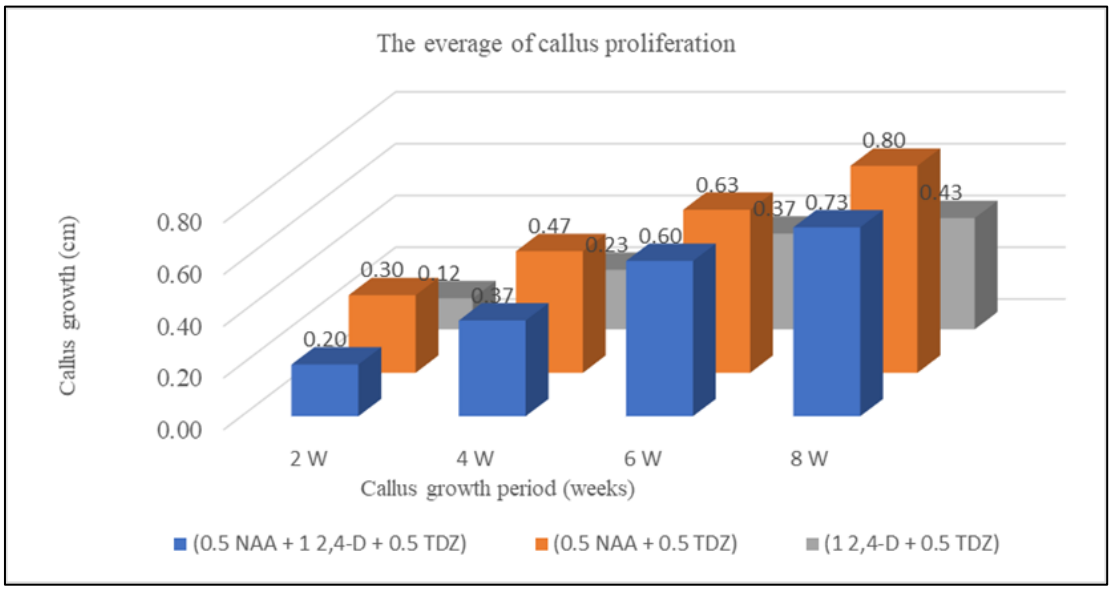


Figure 3 Callus proliferation; callus slow growth in weeks on different combination of auxin and cytokinin (NAA, 2,4-D and TDZ)

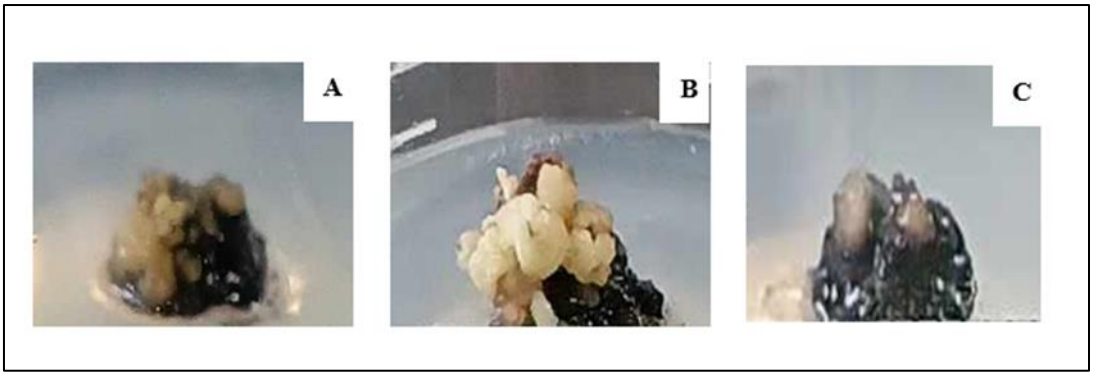


Figure 4 Effect of auxin and cytokinin combination on callus proliferation ½ MS medium supplemented with (A) 0.5 mg/LNAA + 1 mg/L 2,4-D + 0.5 mg/LTDZ; (B) 0.5 mg/L NAA + 0.5 mg/L TDZ and (C) 1 mg/L 2,4-D + 0.5 mg/L TDZ. (Bars indicated 1 cm)

3.3. Shoot induction

Shoot induction was unsuccessful in this procedure, as none of the treatments resulted in shoot development from the various types of calluses. The study used a single medium containing 3 mg/L BAP and 1 mg/L NAA to induce shoots from three types of calluses due to limited callus availability. The results showed that callus type II induced adventive embryonic shoots more effectively than the other types, producing several embryonic shoots that died 12 weeks after being sub-cultured to the medium. Callus type I formed a few single shoots that died 10 weeks after being applied to the shooting medium. Lastly, callus type III did not promote shoot development and only exhibited callus growth. This type of callus took a long time to develop and was eventually sub-cultured to the root induction medium (Figures 5).

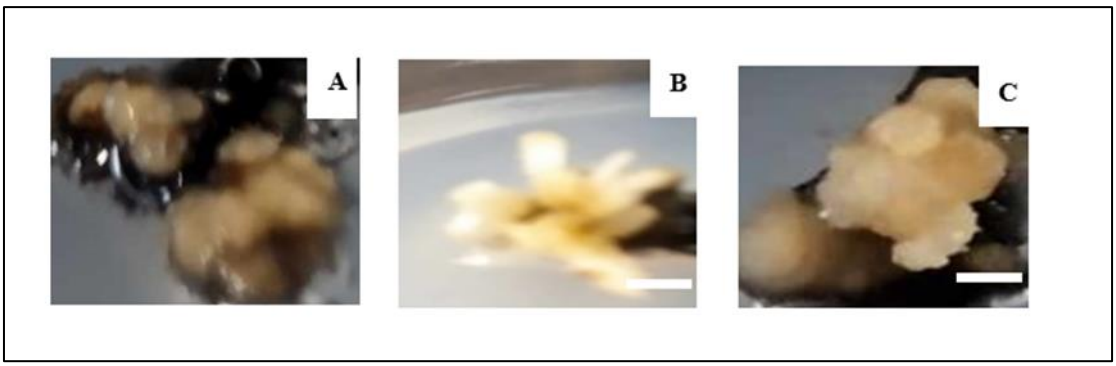
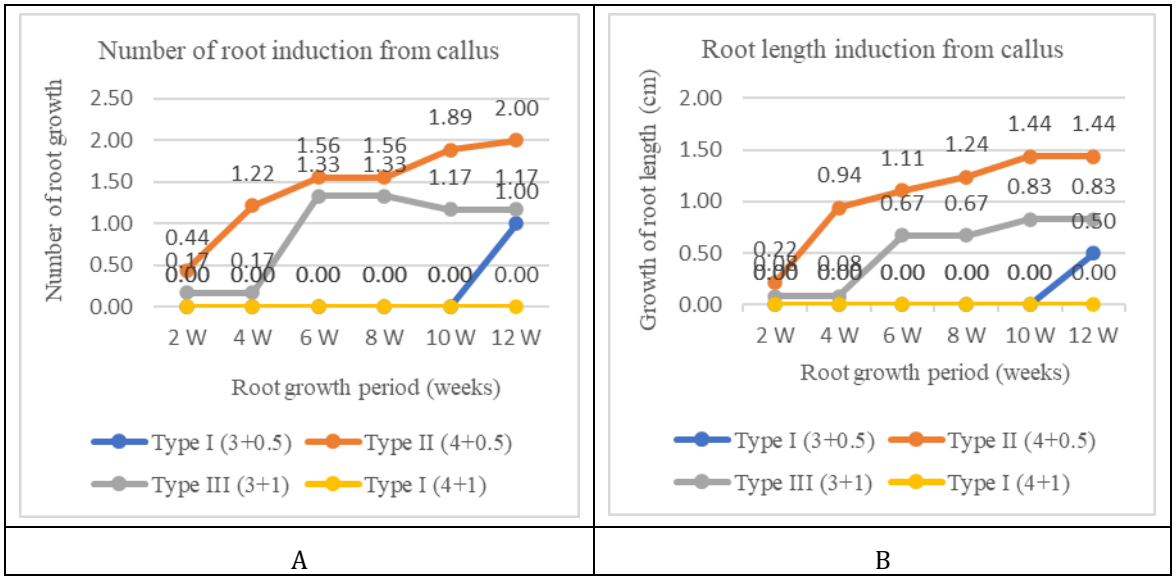


Figure 5 Shoot induction of callus after 8 weeks (A) Callus promoted few embryogenic shoots; (B) Callus generated several embryonic shoots; (C) Callus did not developed any shoot. (Bars indicated 1 cm)

Root induction was carried out after Calli failed to induce shoots. The three callus types were transferred to the rooting medium treated with 0.5 mg/L TDZ + 1 mg/L IBA and growth parameters were investigated over a 12-weeks period. The results showed that callus type II, promoted the highest root formation rate (2.00 roots/explant), followed by callus type III induced a root formation rate of 1.17 roots/explant. Callus type I developed roots very late, taking 10 weeks after subculture to produce roots at a rate of 1.00 root/explant. In terms of root length, callus type II exhibited slight growth, with root length increasing from 2 weeks to 12 weeks, averaging 1.44 cm. Callus type III showed slow initial growth from 4 weeks to 12 weeks, with an average root length of 0.83 cm, while callus type I exhibited root growth after 10 weeks, with an average length of 0.50 cm (Figure 6).



A B

Figure 6 Effect of auxins and cytokinin combination on adventive root formation after subculture to root induction medium supplemented with 0.5 mg/L TDZ + 1 mg/L IBA. (A) Number of root induction (B) Root length development

3.4. Studies on anti-browning and antioxidant of callus induction

The studies revealed that neither white nor yellow callus formation occurred under treatments involving activated charcoal or PVP with ascorbic acid. Specifically, the use of activated charcoal did not result in the formation of black or white callus, while treatments with PVP and ascorbic acid only produced black, or dead, callus. Despite testing various explants of different sizes from both the abaxial and adaxial leaf surfaces, no white callus formation was observed throughout the 16-week evaluation. However, the formation of black or dead callus was noted in the *M. acuminata* cv. Dwarf banana treatment, which utilized a half-strength MS medium supplemented with 2,4-D and BAP, along with PVP and ascorbic acid 200 + 15 g/L (Table 4). This research advances our understanding of the conditions that promote callus induction in explants and provides insights into the potential for white or yellow callus formation.

3.5. Studies on influence of nitrogen and carbon source in callus induction

In vitro conditions demonstrated that the amount of nitrogen significantly affects cell growth and differentiation rates. Numerous studies have reported that reducing the nitrogen source in the MS medium can enhance callus formation. In this study, it was observed that treatments with half-strength nitrogen sources combined with full-strength and reduced sucrose levels did not induce the formation of white callus. Instead, these treatments predominantly resulted in the formation of black or dead callus. Among the cultivars tested over 16 weeks, *Musa* spp. cv. 'Cavendish' exhibited the highest rate of black callus formation when grown in a half-strength macro-element medium supplemented with NAA, TDZ, and 15 g of sucrose. This was followed by the cultivar *Musa x paradisiaca* cv. Ice Cream, which also produced a significant amount of black callus in a half-strength MS medium containing 2,4-D, BAP, and 10.5 g of sucrose (Table 5). This research highlighted the impact of nitrogen sources and sucrose concentrations on callus formation in banana cultivars, provided insights into the conditions that favor the induction of black callus while hindering the formation of white and yellow callus.

Table 1 The influences of auxin and cytokinin combination on callus formation of *M. acuminata* cv. Dwarf banana in a half strength MS medium supplemented with various concentration

Cultivars	**PGRs (mg/L)**					**No. explants**	**No. investigated**	**No. of Callus formation rate (%)**		**Description of callus**		**Remark**
	2,4-D	NAA	KT	BA	TDZ			White/yellow Callus	Black/Dead Callus	Color	Texture	
M. acuminata cv. Dwarf banana	0	0	-	-	-	15	15	0 (0)	0 (0)	-	-	-
	1	0.5	-	-	-	15	11	0 (0)	3 (27.27)	-	-	-
	2	0.5	-	-	-	15	11	0 (0)	3 (27.27)	-	-	-
	3	0.5	-	-	-	101	87	2 (2.30)	21 (24.13)	White	Friable	Growing very slowly
	4	0.5	-	-	-	101	86	5 (5.81)	18 (20.93)	White yellowed	Complex	Growing slowly
	1	1	-	-	-	15	14	0 (0)	1 (7.14)	-	-	-
	2	1	-	-	-	15	15	0 (0)	0 (0)	-	-	-
	3	1	-	-	-	101	93	4 (4.30)	29 (31.18)	White	Hard compact	Growing slowly
	4	1	-	-	-	101	90	2 (2.22)	3 (3.33)	White	Friable	Growing very slowly
	3	-	0.5	-	-	16	9	0 (0)	7 (77.77)	-	-	-
	4	-	0.5	-	-	16	11	0 (0)	4 (36.36)	-	-	-
	3	-	1	-	-	16	12	0 (0)	7 (58.33)	-	-	-
	4	-	1	-	-	16	9	0 (0)	5 (55.55)	-	-	-
	3	-	-	0.5	-	16	8	0 (0)	4 (50.00)	-	-	-
	4	-	-	0.5	-	16	8	0 (0)	4 (50.00)	-	-	-
	3	-	-	1	-	16	10	0 (0)	5 (50.00)	-	-	-
	4	-	-	1	-	16	12	0 (0)	4 (33.33)	-	-	-
	2	-	-	-	0.1	16	11	0 (0)	9 (81.81)	-	-	-
	4	-	-	-	0.1	16	12	0 (0)	9 (75.00)	-	-	-

	2	-	-	-	1	16	**12**	0 (0)	10 (83.33)	-	-	-
	4	-	-	-	1	16	6	0 (0)	3 (50.50)	-	-	-
	-	0.5	-	-	0.5	16	6	0 (0)	0 (0)	-	-	-
	-	1	-	-	0.5	16	6	0 (0)	3 (50.00)	-	-	-
	-	2	-	-	0.5	16	6	0 (0)	0 (0)	-	-	-
	-	0.5	-	-	1	16	6	0 (0)	0 (0)	-	-	-
	-	0.5	-	-	2	16	6	0 (0)	0 (0)	-	-	-

Table 2 The effect of auxin and cytokinin combination on callus formation of *M. luikuiensis*, *M. paradisiaca* cv. Lathundan and *Musa* spp. cv. 'cavendish' in a half strength MS medium supplemented with various concentration

Cultivars	**PGRs (mg/L)**					**No. explants**	**No. investigated**	**No. of Callus formation (%)**		**Description of callus**		**Remark**
	2,4-D	NAA	KT	BA	TDZ			White/yellow Callus	Black/Dead Callus	Color	Texture	
M. luikuiensis	3	0.5	-	-	-	16	15	0 (0)	4 (26.67)	-	-	-
	4	0.5	-	-	-	16	14	0 (0)	3 (21.43)	-	-	-
	3	1	-	-	-	16	15	0 (0)	0 (0)	-	-	-
	4	1	-	-	-	16	15	0 (0)	0 (0)	-	-	-
	3	-	0.5	-	-	16	10	0 (0)	8 (80.00)	-	-	-
	4	-	0.5	-	-	16	12	0 (0)	10 (83.33)	-	-	-
	3	-	1	-	-	16	9	0 (0)	7 (77.77)	-	-	-
	4	-	1	-	-	16	10	0 (0)	6 (60.00)	-	-	-
M. paradisiaca cv. Lathundan	3	0.5	-	-	-	16	14	0 (0)	0 (0)	-	-	-
	4	0.5	-	-	-	16	14	0 (0)	2 (14.29)	-	-	-
	3	1	-	-	-	16	15	0 (0)	0 (0)	-	-	-
	4	1	-	-	-	16	16	0 (0)	0 (0)	-	-	-
	3	-	0.5	-	-	16	15	0 (0)	5 (33.33)	-	-	-

	4	-	0.5	-	-	16	14	0 (0)	5 (35.71)	-	-	-
	3	-	1	-	-	16	15	0 (0)	5 (33.33)	-	-	-
	4	-	1	-	-	16	14	0 (0)	4 (28.57)	-	-	-
Musa spp. *cv. 'cavendish'*	3	0.5	-	-	-	16	15	0 (0)	9 (60.00)	-	-	-
	4	0.5	-	-	-	16	15	0 (0)	7 (46.67)	-	-	-
	3	1	-	-	-	16	13	0 (0)	5 (38.46)	-	-	-
	4	1	-	-	-	16	14	0 (0)	3 (21.43)	-	-	-
	3	-	0.5	-	-	16	11	0 (0)	5 (45.45)	-	-	-
	4	-	0.5	-	-	16	12	0 (0)	7 (58.33)	-	-	-
	3	-	1	-	-	16	12	0 (0)	8 (66.66)	-	-	-
	4	-	2	-	-	16	9	0 (0)	4 (44.44)	-	-	-
	3	-	-	0.5	-	16	11	0 (0)	5 (45.45)	-	-	-
	4	-	-	0.5	-	16	11	0 (0)	7 (63.63)	-	-	-
	3	-	-	1	-	16	14	0 (0)	4 (28.57)	-	-	-
	4	-	-	2	-	16	13	0 (0)	9 (69.23)	-	-	-
	-	0.5	-	-	0.5	16	9	0 (0)	0 (0)	-	-	-
	-	1	-	-	0.5	16	9	0 (0)	0 (0)	-	-	-
	-	2	-	-	0.5	16	9	0 (0)	3 (33.33)	-	-	-
	-	0.5	-	-	1	16	9	0 (0)	0 (0)	-	-	-
	-	0.5	-	-	2	16	9	0 (0)	0 (0)	-	-	-

Table 3 The impact of auxin and cytokinin combination on callus formation of ornamental banana *Musa x paradisiaca* "Ae Ae" in a half strength MS medium supplemented with various concentration

Cultivars	**PGRs (mg/L)**					**No. explants**	**No. investigated**	**No. of Callus formation (%)**		**Description of callus**		**Remark**
	2,4-D	NAA	KT	BA	TDZ			White/yellow Callus	Black/Dead Callus	Color	Texture	
Musa x paradisiaca "Ae Ae"	3	0.5	-	-	-	16	10	0 (0.0)	1 (10.00)	-	-	-
	4	0.5	-	-	-	16	13	0 (0.0)	0 (0.0)	-	-	-
	3	1	-	-	-	16	13	0 (0.0)	2 (15.38)	-	-	-
	4	1	-	-	-	16	14	0 (0.0)	2 (14.28)	-	-	-
	3	-	-	0.5	-	16	15	0 (0.0)	13 (86.66)	-	-	-
	4	-	-	0.5	-	16	13	0 (0.0)	10 (76.92)	-	-	-
	3	-	-	1	-	16	15	0 (0.0)	14 (93.33)	-	-	-
	4	-	-	1	-	16	13	0 (0.0)	13 (100.0)	-	-	-
	2	-	-	-	0.1	16	14	0 (0.0)	13 (92.85)	-	-	-
	2	-	-	-	1	16	13	0 (0.0)	12 (92.30)	-	-	-
	4	-	-	-	0.1	16	13	0 (0.0)	10 (76.92)	-	-	-
	4	-	-	-	1	16	12	0 (0.0)	12 (100.0)	-	-	-
	-	0.5	-	-	0.5	16	6	0 (0.0)	3 (50.00)	-	-	-
	-	1	-	-	0.5	16	6	0 (0.0)	3 (50.00)	-	-	-
	-	2	-	-	0.5	16	6	0 (0.0)	3 (50.00)	-	-	-
	-	0.5	-	-	1	16	0	0 (0.0)	0 (0.0)	-	-	-
	-	0.5	-	-	2	16	0	0 (0.0)	0 (0.0)	-	-	-

Table 4 The influence of auxin and cytokinin concentration with activated charcoal and PVP with ascorbic acid on callus formation of *M. acuminata* cv. Dwarf banana and *Musa* spp. cv. 'cavendish'.

Cultivars	**PGRs (mg/L)**						**No. explant**	**No. investigated.**	**No. of callus formation rate**	
	2.4-D	NAA	BAP	TDZ	Activated charcoal (g/L)	PVP + Ascorbic acid (g/L)			white/yellow Calli (%)	black/dead Calli (%)
M. acuminata cv. Dwarf banana	3	0.5			200		16	11	0 (0)	0 (0)
	4	0.5			200		16	12	0 (0)	0 (0)
	3	1			200		16	8	0 (0)	0 (0)
	4	1			200		16	13	0 (0)	0 (0)
	3		0.5			200+15	16	8	0 (0)	8 (100)
	4		0.5			200+15	16	7	0 (0)	6 (85.71)
	3		1			200+15	16	8	0 (0)	6 (75.00)
	4		1			200+15	16	7	0 (0)	7 (100)
	3			0.5		200+15	16	8	0 (0)	8 (100)
	4			0.5		200+15	16	8	0 (0)	6 (75.00)
	3			1		200+15	16	8	0 (0)	6 (75.00)
	4			1		200+15	16	7	0 (0)	6 (85.71)
	3	0.5				200+15	16	3	0 (0)	0 (0)
	4	0.5				200+15	16	0	0 (0)	0 (0)
	3	1				200+15	16	2	0 (0)	0 (0)
	4	1				200+15	16	0	0 (0)	0 (0)
Musa spp. cv. 'cavendish'	3		0.5			200+15	16	8	0 (0)	7 (87.5)
	4		0.5			200+15	16	8	0 (0)	8 (100)
	3		1			200+15	16	7	0 (0)	7 (100)
	4		1			200+15	16	8	0 (0)	3 (37.5)
	3			0.5		200+15	16	8	0 (0)	6 (75.00)

	4			0.5		200+15	16	6	0 (0)	6 (100)
	3			1		200+15	16	8	0 (0)	6 (75.00)
	4			1		200+15	16	6	0 (0)	4 (6.66)
	3	0.5				200+15	16	3	0 (0)	2 (66.66)
	4	0.5				200+15	16	3	0 (0)	2 (66.66)
	3	1				200+15	16	2	0 (0)	1 (50.00)
	4	1				200+15	16	3	0 (0)	1 (33.33)

Table 5 The effect of combination of auxin and cytokinin with nitrogen and carbon source on callus formation of *Musa* spp. cv. 'cavendish', *Musa paradisiaca* cv. Latundan, *Musa acuminata x balbisiana* (AAB Group) and *Masa x paradisiaca* cv. Ice Cream

Cultivars	**PGRs (mg/L)**		**Sucrose 1/2 strength of MS (g)**	**Sucrose 30% of MS (g)**	**Sucrose 50% of MS (g)**	**No. explant**	**No. investigated**	**No. of Callus formation (rate)**	
	NAA	TDZ						white/yellow Calli (%)	black/dead Calli (%)
Musa spp. *cv. 'cavendish'*	0.5	0.5	15	-	-	16	6	0	1 (16.66)
	1	0.5	15	-	-	16	4	0 (0)	1 (25.00)
	2	0.5	15	-	-	16	8	0 (0)	1 (12.20)
	0.5	1	15	-	-	16	6	0 (0)	3 (50.00)
Musa paradisiaca cv. Latundan	0.5	0.5	15	-	-	16	6	0 (0)	2 (33.33)
	1	0.5	15	-	-	16	12	0 (0)	3 (25.00)
	2	0.5	15	-	-	16	10	0 (0)	4 (40.00)
	0.5	1	15	-	-	16	14	0 (0)	4 (28.57)
Musa acuminata x balbisiana (AAB Group)	0.5	0.5	-	10.5	-	16	16	0 (0)	8 (50.00)
	1	0.5	-	10.5	-	16	16	0 (0)	7 (43.75)
	2	0.5	-	10.5	-	16	16	0 (0)	6 (37.50)
	0.5	1	-	10.5	-	16	16	0 (0)	6 (37.50)
	0.5	0.5	-	-	7.5	16	16	0 (0)	4 (25.00)
	1	0.5	-	-	7.5	16	16	0 (0)	8 (50.00)
	2	0.5	-	-	7.5	16	16	0 (0)	7 (43.75)
	0.5	1	-	-	7.5	16	16	0 (0)	5 (31.25)
Masa x paradisiaca cv. Ice Cream	0.5	0.5	-	10.5	-	16	16	0 (0)	16 (100)
	1	0.5	-	10.5	-	16	16	0 (0)	16 (100)
	2	0.5	-	10.5	-	16	16	0 (0)	14 (87.50)
	0.5	1	-	10.5	-	16	16	0 (0)	14 (87.50)
	0.5	0.5	-	-	7.5	16	16	0 (0)	12 (75.00)
	1	0.5	-	-	7.5	16	16	0 (0)	16 (100)
	2	1	-	-	7.5	16	16	0 (0)	12 (75.00)
	0.5	1	-	-	7.5	16	16	0 (0)	14 (87.75)

4. Discussion

The study of leaf callus induction faced numerous challenges due to several factors. This research identified various conditions that were difficult to achieve. Monocotyledons, such as sugarcane, rice, corn, alocasia, and banana, have a single leaf and fewer parenchyma cells, which promote callus formation (Scarpella, E., and Meijer, A. H. 2004). This may explain why these plants are not commonly propagated through their leaves, and a few reports exist on their leaf propagation. Costa et al., (2009) reported that the leaves of banana plant can induce alterations in tissues linked to photosynthesis, poorly functional stomata, high stomatal density, poorly developed palisade parenchyma. According to this research, Callus formation took a long time, starting between 8 and 16 weeks, with the primary research lasting 19 weeks. During this period, white callus formed under certain treatments, but black callus which considered dead, was more prevalent. Different banana cultivars showed varying callus induction; only Musa acuminata cv. Dwarf banana produced white and yellow callus, which is suitable for shoot and root development. Additionally, Bairu et al., (2006) revealed that the concentration of surface sterilization and plant growth regulators (PGRs) affected callus formation, with many explants forming black callus or becoming contaminated. While some researchers reported that auxin alone could induce callus, this study found that a combination of auxin (2,4-D + NAA) promoted white callus formation. Ather et al., (2009) studies callus induction of sugarcane reported that concentration 3 mg/L 2,4-D induced high Calli forming. Shukla, et al., (2018) also dedicated that applied 4.00 mg/L 2,4–D developed minimum time of callus induction. similarly, to this study combination auxin (2,4-D+NAA) was promoted white callus formation of banana leaf. Type of callus, Transparent water callus was Soft Calli, white colour with smooth, wet, and easy to break into small pieces when touched. Callus This type is formed by a spongy, translucent, white, mushy tissue which result not good for shoot regeneration (Grando, M. F., Franklin, C. I., and Shatters, R. G. (2002). White callus containing small clusters exhibited dense cytoplasm, large vacuoles, and mitochondria, while yellow callus with large clusters was characterized by thick walls and intercellular spaces (Ribeiro, L. D. O., Paiva, L. V., Pádua, M. S., Santos, B. R., Alves, E., and Stein, V. C. (2012). Callus proliferation, an equal combination of auxin and cytokinin resulted in optimal callus growth, consistent with previous research. Increased auxin levels promoted root development, while higher cytokinin levels encouraged shoot formation (Suman, S. 2017). Shoot regeneration from callus combination of BAP and NAA was the best callus in duction of grape's leaf (Khan, N., Ahmed, M., Hafiz, I., Abbasi, N., Ejaz, S., and Anjum, M. 2015). and plant regeneration from callus culture of banana shoot tip (Koarapatchaikul, K., and Kanchanapoom, K. 2010). In contrast, this research callus of banana leaf was leaded to dead. Moreover, environmental conditions like incubation temperature or laminar room conditions influenced callus formation and growth of banana (Rachmi, D., and Purnomo, D. 2020, March).

Browning in cells primarily involves the production of secondary metabolites, which can be mitigated by using low concentrations of charcoal in the medium (Ahmad, I., Hussain, T., Ashraf, I., Nafees, M., Maryam, R. M., and Iqbal, M. (2013). The intensity of callus browning can be reduced by adding a combination of 20-100 mg/L ascorbic acid and 10-50 mg/L citric acid to the medium (Gemechu, E. C., and Amante, G. 2021). According to Gemechu and Amante (2021) , browning is caused by the accumulation and oxidation of phenolic compounds. Incorporating antioxidants such as 0.2-0.5 mg/L PVP and 15-250 mg/L ascorbic acid into the medium can help alleviate this issue. Browning in plant tissue culture refers to the release of brown substances or phenolic compounds from the explant into the medium during dedifferentiation and/or redifferentiation processes. This phenomenon often leads to the formation of brown callus, which is associated with poor growth and ultimately results in cell death (Chaudhary, G., and Dantu, P. K. 2015). Regarding to Shanjani (2003) a study on the effect of nitrogen on callus induction and plant regeneration in *Juniperus excelsa* found that using a medium with half-strength MS nitrogen significantly improved callus production, an effect further enhanced by the addition of glutamine. This finding served as a basis for the present study on banana leaf tissue cultures. However, in the current research, no white callus formation was observed in banana leaf cultures. Despite exploring various nitrogen concentrations and treatments based on Shanjani's findings, the anticipated outcome of white callus production did not occur.

5. Conclusion

The leaf culture method was achieved only callus induction stages due to limited white/yellow calluses forming and needed extensive investigation, a few calluses were formed using a combination of auxins (2,4-D and NAA). only one banana cultivar as M. acuminata cv. Dwarf banana was induced callus. Root induction from callus was successful by utilizing combination of auxin and cytokinin (IBA and IBA) but shoot regeneration was unsuccessful and took a long time to complete the processing. However, this banana culture research would be significant information for future research to advance further.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest

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