Research Article ISSN 2771-9014

Journal of Biotechnology and its Applications

Evaluation of Sucker Category for *In Vitro* Bud Proliferation and Genetic Association of Mother and Surrounding Suckers of *Musa* spp

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Received: 16 Dec 2023; **Accepted:** 25 Jan 2024; **Published:** 01 Feb 2024

Citation: Victor A, Rebecca B, Ruth P, et al. Evaluation of Sucker Category for *In Vitro* Bud Proliferation and Genetic Association of Mother and Surrounding Suckers of Musa spp. J Biotechnology App. 2023; 2(1); 1-10.

ABSTRACT

Commercial production of plantain (Musa spp) is challenging due to insufficient planting materials as vegetative propagation using the same planting materials year after year causes cultivar decline due to pathogen load. Although different types of plantain suckers (maiden, sword and peeper) are available as sources of ex-plant for in vitro propagation, there is a dearth of information on the appropriate type of sucker to be used as source of ex-plants for in vitro initiation and bud proliferation. The purpose of this study was to evaluate the effect of different sucker types of two local plantain cultivars for in vitro establishment, their proliferation performance (bud and shoot number) on Recipient á Immersion Temproraire Automatique (RITA@) temporary immersion bioreactor system (TIBS). This research also assessed the relatedness of sucker types at different developmental stages. Ex-plants obtained from three different sucker types (maiden, sword and peeper) of the two local plantain cultivars Apantu (false horn plantain) and Apem (french plantain) were aseptically initiated on a supplemented Murashige and Skoog's (MS) complete medium while the ex-plant multiplication was done on RITA@ TIBS. Genomic DNA from cigar leaves of the parent plant and suckers derived from the parent plant (maiden, sword and peeper) were also extracted using a modified Cetyl trimethylammonium bromide (CTAB) protocol and analyzed with 15 SSR markers. Analyzed data shows a significant difference ($p \leq 0.01$, $p \leq 0.05$) in ex-plants contamination, the number of leaves and shoots. Sword sucker had the lowest contamination of 0% among the three explants used for initiation. Suckers arising from a parent were more closely related to suckers of the same age than to their parent plant. These advances are expected to boost the proliferation rates of tissue culture plantain for commercial production.

Keywords

Plantain cultivars, Suckers, RITA@ TIBS, Genetic relationship.

Introduction

The *Musa* species, including plantain and banana, is one of the early species cultivated by mankind [1]. Plantain (*Musa paradisiaca* AAB) is consumable only after cooking due to its high starch content even when ripe, unlike banana which is consumable when ripe without cooking. *Musa* species are originally crops of the tropics but have been adapted to a range of climatic conditions currently [1]. The adaptive nature of this species to environmental conditions like drought makes it a valuable crop for food security particularly in rural communities of developing countries. It is also high in calories, essential minerals, and vitamins, and it contains low cholesterol [2]. Plantain production worldwide was 41.11 million MT between 2001 and 2021, and Ghana was among the countries with a high contribution [3]. In Ghana, plantain is ranked third after yam and cassava as the most stable food crop and contributes about 13.1% of the Agriculture's Gross Domestic Product [4,5]. The importance of growing plantain

cannot be underestimated in recent years since plantain is gradually becoming a cash crop [6].

Commercial production of plantain (*Musa* spp) is a challenge as it is propagated vegetatively, and on average, one plant can produce between 5 to 10 suckers in a year [7], which makes commercial production of plantain difficult due to insufficient planting materials since a lot of planting materials are required; 800 to 1000 stands per acre of land [8]. Conventionally, plantain is grown as a semi-perennial crop where the plant is allowed to produce continuous shoots from a subterranean stem and is unable to produce enough planting material for large-scale production. To meet the needs of commercial production and the growing population, tissue culture techniques are adopted in the production of plantain planting materials. *In vitro* micropropagation has a higher multiplication rate of about 10,000 plantlets from one explant within 8 months [9] and provides disease and pathogen-free planting materials that can be established throughout the year [10]. There are different types of suckers differentiated by their developmental stages. The different physiological stages are the peeper sucker (small sucker appearing just above the ground and bearing scaly leaves only), sword sucker (large sucker with lanceolate leaves), and maiden sucker (large non-fruiting sucker with foliage leaves) [9]. Each of these suckers used in micropropagation responds differently in terms of survival, and multiplication rates among others. There is a dearth of information on the appropriate type of sucker to be used as explants for *in vitro* bud initiation and proliferation while maintaining genetic fidelity in the tissue culture derived planting materials.

Routinely, plantain cultures are maintained on a medium supplemented with agar. The use of a liquid medium in bioreactor systems can result in faster multiplication rates that will facilitate meeting the growing demands for clean and high-quality planting materials [11], have confirmed that the Temporary Immersion Bioreactor System (TIBS) generally produces higher planting materials at a lower production cost compared with the semi-solid media.

Currently, little information is available on the genetic relationship between the plantain pseudo-stem and the suckers arising from and surrounding the base of the pseudo-stem, and the possible effects imposed on plantain *in vitro* multiplication. The purpose of this study was to evaluate the effect of different sucker types of two local plantain cultivars for *in vitro* establishment, their proliferation performance (bud and shoot number) on RITA@ TIBS. This research also assessed the relatedness of sucker types at different developmental stages. Therefore, the present study contributes to the improvement of procedures for *in vitro* initiation and proliferation of local plantain cultivars for producing clean planting materials that are genetically uniform for commercial growers.

Methods

The study was carried out at the Tissue Culture and Molecular Biology Laboratories of the Council for Scientific and Industrial Research (CSIR)-Crops Research Institute (CRI), Fumesua in Kumasi, Ghana between January 2022, and January 2023.

Plant Material

Matured and healthy plantain suckers were sampled from plantain fields at Kwadaso Agric College (6.671107, -1.660459) on 31st January 2021 and Fumesua in the Ashanti region on 30th March 2022, in Ghana. The sampling was based on the physiological ages of the suckers. The different sucker types maiden, sword, and peeper were obtained from French plantain (Apem) and False Horn plantain (Apantu) cultivars.

Explant Preparation

Leaves and roots were removed from suckers obtained from the field and layers of the sheath were peeled off. The suckers were trimmed to a length of 1 - 1.5 cm and a diameter of about 2 - 2.5 cm (Figure 1). Explants were separated by treating each in an independent container and thoroughly washed under running water for two minutes to get rid of adhering debris. The trimmed suckers were washed again with liquid soap for two minutes before taking them into a sterilized laminar flow hood. The washed suckers were surface sterilized with 70% alcohol for five minutes and 20% household bleach (with an active ingredient of 3.5% sodium hypochlorite) with a few drops of Tween 20 for 20 minutes. The sterilized suckers were trimmed to a length of 1-2 cm, sterilized again with 10% household bleach with the same active ingredient of 3.5% sodium hypochlorite for 10 min. The suckers explants were washed after each sterilization with sterilized distilled water

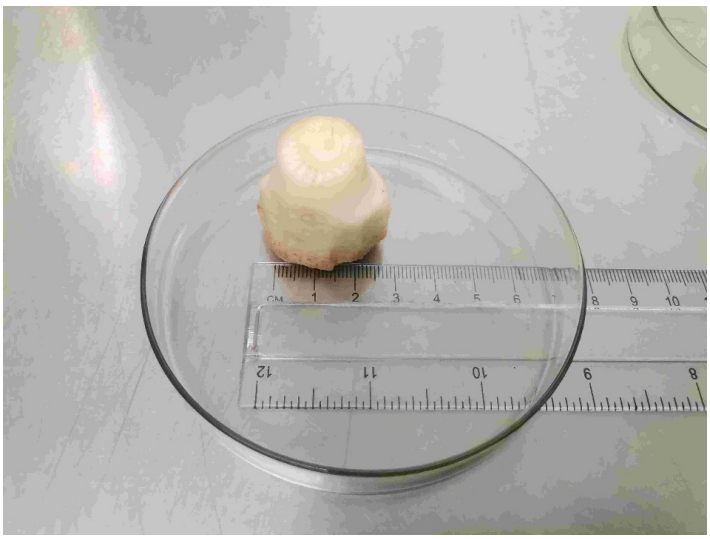

Figure 1: Trimmed sucker for surface sterilization.

Experimental setup

The experiment was a 2 x 3 factorial treatment structure laid in a Completely Randomized Design (CRD) with three replications. The two factors were the cultivars with two levels (Apem (French plantain) and Apantu (false horn plantain)) and the sucker type with 3 levels (peeper, sword and maiden suckers).

Media Preparation

Murashige and Skoog's (MS) Complete Medium (PhytoTech Labs, Inc., Lenexa KS) [12] was prepared and supplemented with 3% sugar, ascorbic acid (0.01 g/l) benzylaminopurine (BAP) (5 x 10^{-5}M), and indole-3-acetic acid (IAA) (1 x 10^{-6}M). After the addition of the required volumes of plant growth regulators, the solution was brought to volume with autoclaved de-ionized water, maintaining the pH of the solutions at 5.7 ± 0.1. Five grams of agar (Sigma-Aldrich, Missouri) were added per liter of media prepared to solidify it. The media with agar was heated for five minutes using a microwave (Samsung, GE107Y-S, Malaysia) oven to melt the agar. Media with agar was dispensed into 25 mm test tubes in aliquots of 10 ml, covered and sterilized at 121°C and 103.42 kPa for 15 minutes using an autoclave. The autoclaved media were allowed to cool in the transfer area of the Tissue Culture Laboratory before use.

The preparation of the multiplication media followed the same steps as the initiation media except for the addition of agar for solidification as liquid media is required for multiplication on RITA@ TIBS.

Initiation, Multiplication and Incubation

Thirteen trimmed and surface sterilized explants of maiden, sword and peeper suckers for both Apem (French plantain) and Apantu (false horn) were established on semi-solid initiation media and sub-cultured twice onto new semi-solid initiation media at four-week intervals. Contaminated explants at the end of each cycle (four weeks/cycle) were discarded and survived explants without contamination (41 and 78 explants of Apem and Apantu respectively) after the initiation stage were cleaned off darkened layers by trimming to remove dead and darkened sheaths and then manipulated by making incisions at the top to break apical dominance by disturbing the apical meristem. These explants were used as the initial explants for the TIB system and the multiplication stage. RITA@ TIBS were filled with 250 ml of MS multiplication liquid media. Each explant of each cultivar was cultured in an independent RITA@ system. Cultures were maintained in a growth room under a temperature of 25 ± 1 ℃ and photoperiod with 16 hours of light photoperiod provided by white fluorescent bulbs (Philips T8 LED, 1700 lm, 18 Watt) and 8 hours of darkness for four cycles of four weeks each. The multiplication on the RITA@ TIBS was programmed with an immersion cycle of two minutes of flush time and two hours of rest which involved the flooding of explants for 2 minutes every 2 hours with an electronic timer. At every sub-culture, explants without contamination were cleaned, split to injure the apical meristem for the induction of bud proliferation and transferred onto a new media for another growth cycle. Initiated cultures that exhibited slow growth were transferred to fresh media. After the four cycles of subculturing, explants were introduced onto a multiplication media with half the amount of BAP (2.5x10^{-5} M) used in the previous multiplication media to induce the formation of well-defined shoots.

Data Collection

Data was collected on culture survival and contamination percentages. The number of buds, number of leaves, bud dimension, number of shoots, and average shoot height were also taken for analysis.

Statistical Analysis

Data collected was subjected to analysis of variance (ANOVA) and means were separated using the honest significant difference (HSD) at a 5% significance level. Count and percentage data were transformed to conform to the assumptions of ANOVA analysis by making the data assume a normal distribution. A correlation analysis between contamination and survival percentage was done. The statistical package used for the analysis was R version 4.2.2.

Molecular Characterization

Plantain suckers were selected based on colonies. A colony is made up of all suckers of the four different developmental stages (fruiting sucker, maiden sucker, sword sucker, and peeper sucker). Such colonies were selected from two cultivars (Apem (French plantain) and Apantu (false horn) (as described in Table 1). Six colonies each of Apem and Apantu were selected for the study. Cultivars and sucker stages were selected based on known morphological data. Leaf samples were collected from young cigar leaves of each sucker of each colony selected for both cultivars. The leaves were collected with a pair of forceps into an Eppendorf tube and stored in liquid nitrogen during transportation to the laboratory. Young cigar leaves of an approximate weight of 0.2 g previously stored in liquid nitrogen were grounded with liquid nitrogen and genomic DNA was extracted using a modified CTAB (Cetyl trimethylammonium bromide) DNA extraction protocol [13]. A volume of 800 µl lysis buffer (20 mM Tris HCl, 50 mM EDTA, 2 M NaCl, 2 % CTAB, 3 % β-mercaptoethanol) was added to the homogenized sample, followed by 800 µl Phenol:Chloroform:Isoamyl alcohol (25:24:1). The mixture was gently mixed by inversion until it turned milky. The mixture was incubated on ice for 5 mins and centrifuged at 13,000 rpm for 15 mins. The supernatant was transferred into a new 2 ml Eppendorf tube without disturbing the middle layer. Eight hundred microliters of Isopropanol were added, gently mixed then incubated at -20 ℃ overnight. The samples were centrifuged at 10,000 rpm for 10 mins. The nucleic acid was washed with 80 % ethanol and the pellets were dried at room temperature. The DNA pellets were dissolved in 500 µl low-salt Tris EDTA (TE) buffer. RNase (10 µl) was added, mixed gently, and incubated for 45 mins at 37 ℃. 7.5 M Ammonium acetate (250 µl) was added to the samples and incubated on ice for 5 mins and centrifuged for 10 mins at 13,000 rpm. The supernatant was then transferred into a newly labelled 1.5 ml Eppendorf tube and 700 µl of Isopropanol was added and mixed by inversion and centrifuged again for 5 mins at 1,000 rpm. The nucleic acid was washed with 80 % ethanol and the pellets were dried at room temperature. DNA pellets were dissolved in 1 µl low-salt Tris EDTA (TE) buffer. The quantity and quality of DNA extracted were estimated using a Nanodrop spectrophotometer (NanoDrop lite, ThermoScientific) and 0.8 % agarose gel in Tris-acetate EDTA (TAE) buffer respectively. The

DNA separated in agarose gel was visualized with an ultra-violent trans-illuminator (ProteinSimple).

Table 1: *Plantain suckers and varieties sampled for analysis.*

Sample Code	Type Sucker	Local name	Genotypic group	Colony No.
AP-1	Fruiting Sucker (mother plant)	Apantu	False horn	Colony 1
MSAP 1	Maiden Sucker	Apantu	False horn	
SSAP 1	Sword Sucker	Apantu	False horn	
PSAP 1	Peeper Sucker	Apantu	False horn	
AP-2	Fruiting Sucker (mother plant)	Apantu	False horn	Colony 2
MSAP 2	Maiden Sucker	Apantu	False horn	
SSAP 2	Sword Sucker	Apantu	False horn	
PSAP 2	Peeper Sucker	Apantu	False horn	
AP-4	Fruiting sucker (mother plant)	Apantu	False horn	Colony 3
MSAP 4	Maiden sucker	Apantu	False horn	
SSAP 4	Sword sucker	Apantu	False horn	
AP-5	Fruiting sucker (mother plant)	Apantu	False horn	Colony 4
MSAP 5	Maiden sucker	Apantu	False horn	
SSAP 5	Sword sucker	Apantu	False horn	
PSAP 5	Peeper sucker	Apantu	False horn	
AP-6	Fruiting sucker (mother plant)	Apantu	False horn	Colony 5
MSAP 6	Maiden sucker	Apantu	False horn	
SSAP 6	Sword sucker	Apantu	False horn	
PSAP 6	Peeper sucker	Apantu	False horn	
A-1	Fruiting sucker (mother plant)	Apem	French plantain	Colony 6
MSA 1	Maiden sucker	Apem	French plantain	
PSA 1	Peeper sucker	Apem	French plantain	
A-2	Fruiting Sucker (mother plant)	Apem	French plantain	Colony 7
MSA 2	Maiden Sucker	Apem	French plantain	
SSA 2	Sword Sucker	Apem	French plantain	
PSA 2	Peeper Sucker	Apem	French plantain	
A-3	Fruiting sucker (mother plant)	Apem	French plantain	Colony 8
MSA 3	Maiden sucker	Apem	French plantain	
SSA 3	Sword sucker	Apem	French plantain	
PSA 3	Peeper sucker	Apem	French plantain	
A-4	Fruiting sucker (mother plant)	Apem	French plantain	Colony 9
MSA 4	Maiden sucker	Apem	French plantain	
SSA 4	Sword sucker	Apem	French plantain	
PSA 4	Peeper sucker	Apem	French plantain	
A-5	Fruiting sucker (mother plant)	Apem	French plantain	Colony 10
MSA 5	Maiden sucker	Apem	French plantain	
PSA 5	Peeper sucker	Apem	French plantain	
A-6	Fruiting sucker (mother plant)	Apem	French plantain	Colony 11
MSA 6	Maiden sucker	Apem	French plantain	
SSA 6	Sword sucker	Apem	French plantain	
PSA 6	Peeper sucker	Apem	French plantain	

Polymerase Chain Reaction (PCR)

A total of 18 Simple Sequence Repeats (SSR) primer pairs [14] were reconstituted with nuclease-free sterile water (NFSW) to obtain a 100 µM stock solution. Primers were diluted by adding 90 µl of NFSW to 10 µl of each primer to obtain a working stock (10 µM) and stored at -20 ℃. All 18 SSR primers were validated using 10 % of the total samples at the required annealing temperatures (Table 2) for each primer. The PCR samples for the primer validation were prepared with 2.5 µl of NFSW, 5 µl of 2 X DreamTaq Hotstart kit, 0.25 µl each of 10 µM forward and reverse primers and 2 µl of 50 ng DNA template. The PCR amplification was performed at 94 ℃ for 5 mins for the initial denaturation and 35 cycles of denaturation at 94℃ for 45 seconds at required annealing temperatures (Table 2) for 1 minute, and initial extension at 72 ℃ for 1 minute with a final extension at 72 ℃ for 5 mins. Fifteen SSR primers were selected based on reliability, polymorphism, and specificity to the target region and were used to screen 45 plantain suckers from two (Apem (French plantain) and Apantu (false horn), Table 1), as three leaf samples were eliminated. The PCR samples for amplification were performed in a 96-well thermal cycler (Gene AmpPCR System, 9700, AB Biosystems). The PCR products were loaded on a 6 % Polyacrylamide gel with Filtered Autoclaved Distilled Water (FADW), 10X Trix Boric EDTA (TBE), 40 % acrylamide, 10 % Ammonium Per Sulphate (APS) and Tetramethylethylenediamine (TEMED). Electrophoresis was done in a Tris-Boric-EDTA (TBE) buffer for 50 mins at 80 Volts. After electrophoresis, the gel was stained with 5 % ethidium bromide solution and a ProteinSimple transilluminator was used to view the stained gel under UV light. A 50 bp ladder (BioLabs) was used as a reference to compare and estimate DNA fragments' sizes.

Table 2: *SSR primers and sequences used for the study.*

Marker	Primer's sequence (5'-3')	Annealing Temp (℃)	Expected band Size (bp)
MMACIR03	F: TGACCCACGAGAAAAGAAGC	55	110-147
	R: CTCCTCCATAGCCTGACTGC		
MMACIR07	F: AACAACTAGGATGGTAATGT-GTGGAA	53	136-195
	R: GATCTGAGGATGGTTCT-GTTGGAGTG		
	R: ATTAGATCACCGAAGAACTC		
MMACIR39	F: AACACCGTACAGGGAGTCAC	52	329-390
	R: GATACATAAGGCAGTCA-CATTG		
MMACIR40	F: GGCAGCAACAACATACTAC-GAC	54	164-247
	R: CATCTTCACCCCCAT-TCTTTTA		
MMACIR45	F: TGCTGCCTTCATCGCTACTA	57	272-318
	R: ACCGCACCTCCACCTCCTG		
MA3_90	F: GCACGAAGAGGCATCAC	53	147-191
	R: GGCCAAATTTGATGGACT		
MMACIR150	F: ATGCTGTCATTGCCTTGT	54	253-376
	R: GAATGCTGATACCTCTTTGG		
MMACIR152	F: CCACCTTTGAGTTCTCTCC	54	147-195
	R: TTTCCCTCTTCGATTCTGT		

MMACIR164	F: AAGACAAGTTCCATTGCTTG	55	255-458
	R: GTTCGGGCTTTCGGT		
MMACIR196	F: GCTCCAAACCTCCCTTT	55	163-201
	R: CGATGCCACACTGGAC		
MMACIR231	F: GCAAATAGTCAAGGGAATCA	55	236-286
	R: ACCCAGGTCTATCAGGTCA		
MMACIR260	F: GATGTTTGGGCTGTTTCTT	55	194-264
	R: AAGCAGGTCAGATTGTTCC		
MMACIR264	F: AGGAGTGGGAGCCTATTT	53	234-383
	R: CTCCTCGGTCAGTCCTC		
MMACIR307	F: AGACTTGTATCGCTTGGTAAA	54	143-172
	R: ACGCTGCACCAGTCAA		

Source: [14]

Bands were scored as '1' for present and '0' for absent based on the expected band sizes. A cluster analysis was performed on the binary data scored using a neighbour-joining algorithm using an unweighted pair group method with arithmetic average (UPGMA) in Darwin Version 6.0.21. Genetic diversity parameters such as major allele frequency (Na), allele number (Ne), gene diversity (He), and Polymorphic Information Content (PIC) were determined with the [15] method using PowerMarker Software Version 3.25. PCSA and the analysis of molecular variance (AMOVA) were done using GenAlex software Version 6.5.1b2.

Results and Discussion

Initiation and Multiplication Data Analysis

The study compared the growth performance (survival rate, bud and shoot) of two plantain cultivars initiated and multiplied *in vitro* using three different types of suckers (maiden, sword, and peeper suckers) on RITA@ TIBS and assessed the genetic relationship between a parent plant and its surrounding suckers. Table 3 shows results for a two-way ANOVA of the effects of cultivars, explants and their interaction on percent survival and contamination. There were significant differences between cultivars for both percent survival and contamination as well as between the sucker types for percent contamination. The interaction between cultivar and explant type had no significant effect on the percent survival. The Apantu (false horn plantain) cultivar showed the least contamination of 28% between the two cultivars whereas the sword sucker recorded the lowest contamination of 0% among the three sucker types used for the initiation/establishment stage. Meanwhile, maiden suckers were the most contaminated sucker type for both cultivars recording 56% and 90% contamination rates for Apantu (false horn plantain) and Apem (French respectively in the initiation stage (Figure 2). The contamination rates recorded showed a strong negative (-0.89) to the survival rates recorded in the study (Table 4). Fewer explants survived in the initiation phase due to high contaminations which could be attributed to the endophytic microbes within the explants as explained by [16]. Contamination in plant tissue culture is associated with either external or internal factors, while external factors are attributed to inadequate sterilization and other laboratory practices, internal factors are a result of the endophytic microbes found within the explants. Internal microbes cannot be removed with surface sterilization protocols. Contamination in tissue culture leads to death and loss of explants, according to [17], it can lead to about 90% loss of materials. It is expected that matured explants like maiden suckers will accumulate more microbes than younger explants like sword and peeper suckers as similarly observed by [18], in mango, where seeds from ripe mango fruits had higher contamination rates than seeds from unripe mango fruits although both had undergone the same sterilization procedure for micro propagation. This difference was a result of increased microbiological load in the matured fruits which made its seeds highly prone to contamination. Contamination in ripe mango explants resulted in a lower survival rate of 28% compared to seeds from immature mango fruits which produced a high survivability rate between 74.6% and 94.6%. Similarly, in this study, the maiden sucker which is the matured sucker among the three sucker types used recorded the highest contamination (89.9%) and was the least survived among the other suckers although all suckers were surface sterilized with the same procedure. This could be as a result of accumulated endophytic microbes in the maiden sucker. A higher survival rate recorded in sword suckers is consistent with the results of [11], who recorded a 100 % survival rate after three subcultures at 21 days intervals of sword suckers on a semi-solid media.

Table 3: *Analysis of variance at 1% and 5% significance level for percent survival and contamination.*

	Df	F-Value %Survival	F-Value %Contamination
Cultivar	1	8.33**	8.633**
Explant	2	2.17	9.36**
Variety* Explant	2	0.11	1.76

*** $p \leq 0.01$; ** $p \leq 0.05$

Table 4: *Pearson Correlation between Square Roots of Survival (percentage) and contamination.*

	Survival	Contamination
Survival	1	-0.893***
Contamination		1

***$p \leq 0.01$

In the multiplication stage, a significant difference was recorded in leaf and shoot number according to the cultivar type whereas a significant difference in shoot height was as a result of the cultivar and explant interaction (Table 5). Apantu generated 260 leaves and 1268 shoots while Apem generated 59 leaves and 382 shoots (Figures 3A, 3B). Also, the peeper sucker of Apantu recorded the highest average height of 26.4 cm and was significantly different from the peeper suckers of Apem (Figure 3). The difference in shoot numbers observed between the two cultivars could be attributed to the unequal number of explants of the cultivars that were available for the multiplication stage on RITA@ TIBS. Apem lost most of its explants during the initiation stage which reduced the number of explants that were available for this stage. At the beginning of the multiplication stage, Apantu had 88 explants whereas Apem had 44 explants. Also, the difference in leaf and shoot numbers among the cultivars could suggest that the level of cytokinin in the multiplication media influences shoot and leaf formation differently in both cultivars. As the cytokinin level was optimum for bud proliferation in Apem, it caused high leaves

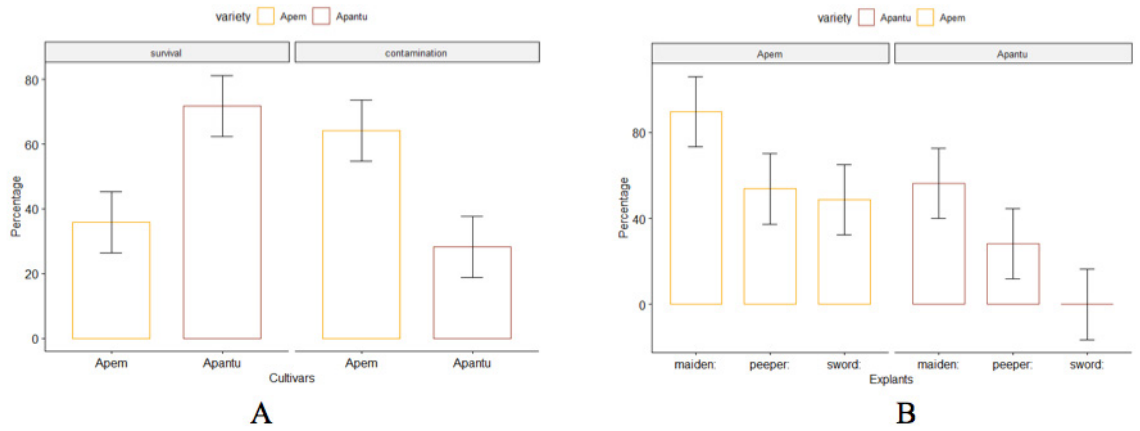

Figure 2: Bar graphs of means for percent (A) contamination and survival of Apem and Apantu and (B) three explants obtained from maiden, sword and peeper suckers after initiation.

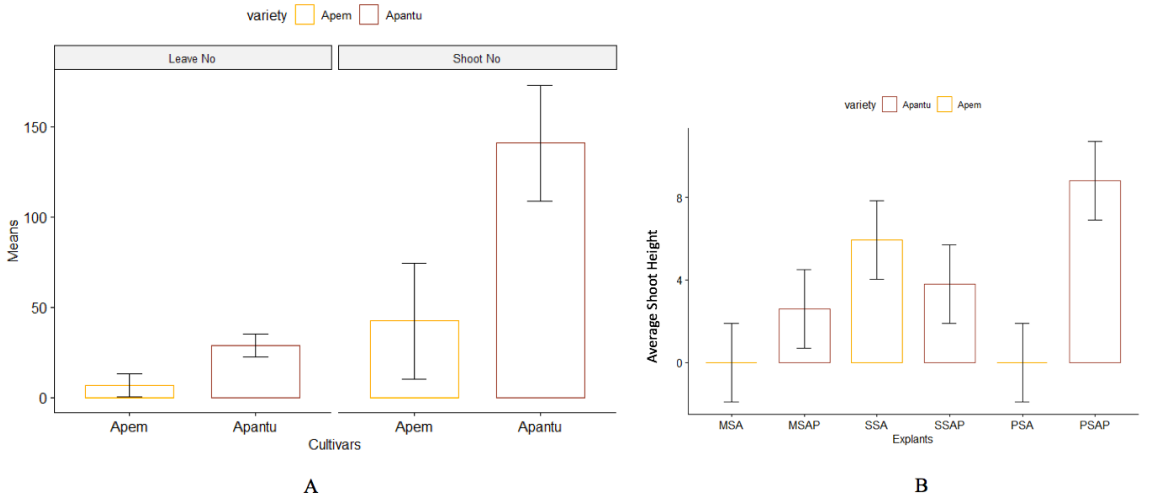

Figure 3: Bar graphs of (A) total number of leaves, total number of shoots and (B) average shoot height for Apem and Apantu initiated and multiplied in vitro using maiden, sword and peeper suckers.

and shoot development in Apantu (Figure 4). This corresponds with the findings of [19] that the combinations of cytokinin and auxin for higher shoots in different plantain varieties differ, also the different growth patterns among plantain cultivars differ in leaf formation among the two cultivars used in this study. Apantu matures faster than and produces shoots and leaves earlier than Apem which could have also influenced the differences in leaf and shoot number. Meanwhile, there was no significant difference in the bud size and number. Irrespective of the difference in the number of explants for the cultivars used in the multiplication stage, no significant differences were observed ($p \geq 0.05$) among bud number and size which confirms the findings made by [11] that multiplication on RITA@ TIBS produces higher bud quantity. Fewer starting materials could therefore generate a higher number of buds on RITA@ TIBS. Although the ANOVA showed no significant difference in bud number among the explant types, every bud generated in tissue culture is a relevant gain. The sword and peeper suckers showed higher bud proliferation than the maiden suckers which agrees with the records of [20], younger explants have greater totipotency potential due to the dense cytoplasm and prominent nucleus in their meristematic centre which increases bud proliferation. Conventionally, the maiden sucker is often used for propagation because it has more reserves and high vigour, thus reducing the growing cycle and increasing yield. Sword and peeper suckers, on the other hand, are less preferred conventionally because it has fewer reserves and less vigour [9]. This seems not to be a hindrance *in vitro* as in this study, the sword and peeper suckers were better explant types for plantain *in vitro* multiplication. The result observed confirms the conclusions made by [21], the age of an explant is not relevant in the number of shoots obtained during micropropagation. The multiplication parameters were positively correlated, with a correlation coefficient range of 0.73-0.96 ($p \leq 0.01$) (Table 6). There was a very strong relationship between the buds and shoot numbers as well as between the bud size and bud number with a correlation coefficient of 0.92 ($p \leq 0.01$) and 0.85 ($p \leq 0.01$) respectively. The strong correlation observed among the bud size, bud and shoot numbers was expected as explained by [22] that shoots are stimulated from axillary bud growth during micro-propagation. Therefore higher bud numbers result in larger bud size which produces higher shoot numbers.

Table 5: *Analysis of variance at 1% and 5% significance level for multiplication responses.*

	D.F	Bud Dimension	Number of Buds	Number of Leaves	Number of Shoots	Total Average Shoot
Variety	1	6.98	3.28	19.86**	9.27**	3.90
Ex-Plant	2	1.26	3.02	1.71	2.96	2.91
Variety*Explant	2	4.00	3.10	4.05	3.35	5.94**

*** $p \leq 0.01$; ** $p \leq 0.05$

Figure 4: Bud proliferation of sword suckers of varieties after the multiplication phase.

Table 6: *Pearson's Correlation between Square Root of Multiplication Responses*

	Bud Dimension	**Number of Buds**	**Number of Leaves**	**Number of Shoots**	**Total Average Shoot**
Bud Dimension	1	0.852***	0.859***	0.811***	0.865***
Number of Buds		1	0.732***	0.918***	0.905***
Number of Leaves			1	0.830***	0.770***
Number of Shoots				1	0.957***
Total Average Shoot					1

*** $p \leq 0.01$;

Genetic Relationship Analysis

This study used 15 SSR primers to characterize two plantain cultivars (Apem (french plantain) and Apantu (false horn plantain), 45 samples in total were genetically analyzed. This analysis hypothesised that the cultivars used will have a narrow genetic diversity and all surrounding suckers will be genetically related to their parent suckers. The 15 SSR markers used produced 389 alleles in total with a minimum of 9 alleles, a maximum of 33 alleles and an average of 25.9 alleles (Table 7). Primer Mm 40 recorded the lowest allele number, and Mm 164 recorded the highest allele number. The expected heterozygosity ranged between 0.82 and 0.93. The Polymorphic Information Content recorded an average of 0.93, with primer Mm 40 recording the lowest of 0.9 and the highest of 0.97 being recorded by Mm 164 (Table 7). The genetic relationship among the 45 plantain samples was estimated with a genetic similarity coefficient range of 0.83 – 0.94, with the most similar samples (0.94) being AP4 and AP5 (Appendix A). A UPGMA analysis grouped the plantain samples according to the cultivars; Apem and Apantu, and also according to the age of the suckers; fruiting/parent suckers -A/AP (cluster 1), maiden – MSA/MSAP (cluster 4 and 5), sword – SSA/SSAP (cluster 3 and 6), and peeper – PSA/PSAP (cluster 2 and 7) suckers (Figure 5), although, some suckers interlaced with unexpected cultivars or physiological age groups. Apem and Apantu parent suckers clustered strictly by cultivars at a similarity coefficient between 0.84 and 0.94 with one parent sucker of Apem interlacing closely with the Apantu parent suckers at a similarity coefficient between 0.88 and 0.89. There was a 0.83 – 0.87 similarity between the maiden suckers of Apem and Apantu and the individual maiden suckers of both Apem and Apantu were closely related at 0.85 – 0.91. Two maiden suckers of Apantu interlaced with peeper suckers of Apem at a similarity coefficient of 0.86 and 0.9 whereas one maiden sucker of Apem was 0.89 similar to the sword suckers of Apem. The sword suckers of Apem and Apantu were generally similar with a coefficient between 0.83 – 0.87 and the individual suckers within each cultivar were similarly related to each other at 0.83 – 0.93. All the sword suckers clustered with their expected variety and physiological age groups. Peepers of Apem and Apantu separated according to cultivars at a similarity coefficient of 0.82 – 0.87 whiles the individual suckers within each cultivar were closely related at 0.85 – 0.92 to each other. All the peeper suckers also clustered with their respective cultivars and physiological age groups with no unexpected clustering (Figure 5). The results demonstrate a narrow genetic distance between the plantain cultivars which is similar to the findings of [23,24]. in which they both stated that there is diversity among plantain cultivars but the existing diversity is narrow. In this study, genetic differences exist among plantain suckers from a single colony, that is, between a plantain parent/fruiting sucker and its surrounding suckers (maiden, sword and peeper suckers). The parent suckers and their surrounding suckers in both cultivars were unexpectedly 83% - 89% related while the same physiological age-specific suckers (maiden, sword and peeper suckers) showed higher similarity up to 94% within the groups. This is similar to the findings in the Henequen plant by [25] where there was genetic similarity between parent plants and daughter rhizomes. An Amplified Fragment Length Polymorphism (AFLP) analysis showed the parent plant and their daughter rhizomes were unidentical but showed similarity at the population level where plants from the same generation population clustered together. These results were repeated to rule out the possibility of an artefact of the technique used, it was therefore concluded that the plants were more related to a generational population than to their parent which demonstrates there is an introduction of genetic variability through asexual reproductive mechanism. A similar result was recorded by [26], in an *in vitro* study with *Musa* spp, the sword suckers from specific parent plants were cultured and the resulting planting materials were analysed for genetic fidelity. The findings of this work indicated an extremely high rate of variation among plants generated from a single plantain meristem using Random Amplified Polymorphic

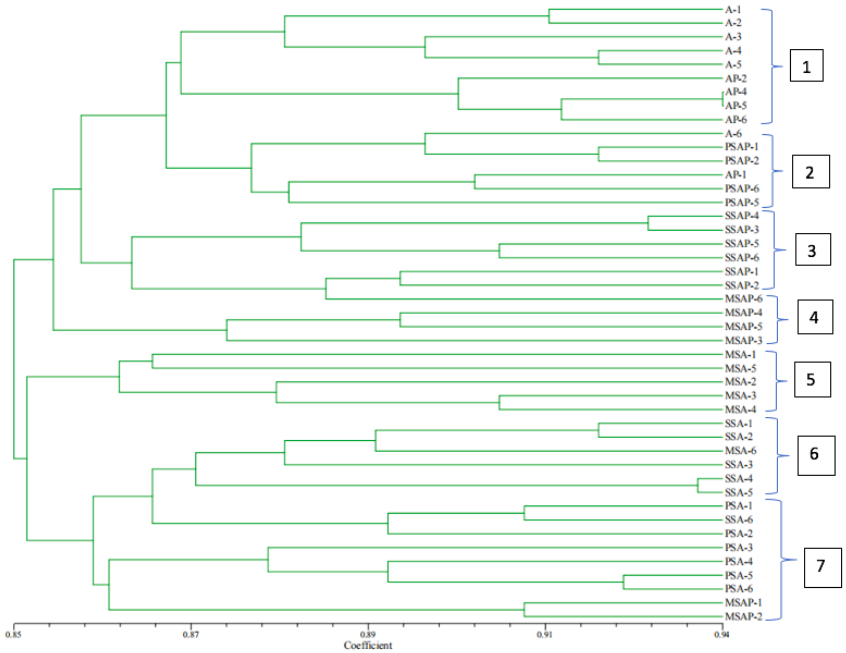

Figure 5: UPGMA cluster analysis showing genetic relationship of the 45 plantain samples generated from NTSYS

DNA (RAPD) markers. The possibility of the variations observed being associated with culture-induced variations in their study was ruled out due to the high frequencies of mutations recorded and rather suggested that the differences reflected genetic differences associated with the original meristem. The clustering of suckers according to the physiological ages recorded in this study could be associated with the SSR markers used. These markers may be age-influenced markers hence demonstrating variations between the parent sucker and their surrounding suckers. This conforms with the findings of [27] in which SSR markers were used to differentiate among individual *Musa* spp with specific traits. They concluded from their study that, SSR markers are efficient in identifying variants and explaining the underlying mechanism behind the resulting variation. From a conclusion made by [23], the phenotypic traits are not enough to predict the genotypic traits of plantain as these two traits do not correlate. The observations made from this study can also be associated with cyclophysis which is a process of ontogenetic ageing as explained by [28]. It is genetically programmed, localized in meristems, accelerates by improved growth conditions and is difficult to reverse. The process is more associated with apical meristems because it produces most of the total growth. According to a study by [29], *a* black locust tree propagated with materials of different ages obtained from one mother plant showed significant differences in growth, leaf traits and physiological traits as well as gene expressions. This led them to relate the inconsistencies to cyclophysis [30]. Explained that reproductive maturity occurs in primary apical and lateral shoot meristems and not in differentiating tissues. The chronological age of a given meristem at a time of transition is therefore not fixed. A tree could simultaneously produce juvenile tissues near the base (while the meristem was young) and produce distal shoots and buds in the adult phase (after the meristem phase change). It can therefore be hypothesised that plantain fruiting suckers produce suckers with varying genetic composition influenced by the maturity stage of the fruiting sucker. A new sucker produced at a younger stage of the fruiting sucker will differ from another sucker generated at a matured stage of the fruiting sucker. More research will be required to confirm the theory of cyclophysis in *Musa* spp as most records of the phenomenon to the best of my knowledge are in tree and shrub spp.

Table 7: *Summary statistics on the SSR markers analysed with PowerMarker Version 3.5.*

Marker	Major Allele Frequency	Sample Size	No. of obs.	Allele No	Gene Diversity	PIC	Effective number of alleles
Primer Mm03	0.20	45.00	45.00	26.00	0.93	0.93	14
Primer Mm 07	0.11	45.00	45.00	28.00	0.95	0.95	21
Primer Mm 24	0.16	45.00	45.00	22.00	0.92	0.92	13
Primer Mm 39	0.07	45.00	45.00	33.00	0.96	0.96	28
Primer Mm 40	0.29	45.00	45.00	9.00	0.82	0.80	6
Primer Mm 45	0.07	45.00	45.00	33.00	0.96	0.96	27
Primer Mm 90	0.09	45.00	45.00	24.00	0.95	0.94	19
Primer Mm 150	0.09	45.00	45.00	27.00	0.95	0.95	21
Primer Mm 152	0.09	45.00	45.00	33.00	0.96	0.96	27
Primer Mm 164	0.04	45.00	45.00	40.00	0.97	0.97	37
Primer Mm 196	0.18	45.00	45.00	18.00	0.90	0.90	10
Primer Mm 231	0.13	45.00	45.00	20.00	0.93	0.93	14
Primer Mm 260	0.13	45.00	45.00	27.00	0.95	0.95	19
Primer Mm 264	0.09	45.00	45.00	29.0000	0.96	0.95	22
Primer Mm 307	0.18	45.00	45.00	20.00	0.92	0.92	13

Conclusion

The purpose of this study was to evaluate the effect of different explant types for two local plantain cultivars for *in vitro* establishment, their proliferation performance (bud and shoot number) on RITA@ TIBS and also the relatedness of the explants at different developmental stages. It can be confirmed from the study that, the sword explant was the best explant source for *in vitro* establishment of both cultivars (Apem (French plantain) and Apantu (false horn)) due to the less microbial load associated with the sucker type. All explants used for the study had good proliferation (buds numbers) on RITA@ TIBS but varying establishment performance which could affect subsequent steps of the tissue culture procedures. For both cultivars used for this study, the sword sucker is recommended as the best explant type for plantain *in vitro* establishment and bud proliferation on RITA@ TIBS since it had high establishment and bud proliferation rates in the initiation and multiplication stages respectively. The cultivars used for the study were closely related with a narrow genetic diversity between them. The suckers used were closely related by physiological age rather than to their respective parent suckers. Further analysis is required to rule out limitations imposed by the markers used to avoid misleading decisions for building breeding strategies and the possibility of cyclophysis in *Musa* spp.

Acknowledgments

The authors wish to express their sincere thanks to scientists, technicians and national service personnel at the CSIR-Crops Research Biotechnology Laboratory who contributed in one way or the other in experimental set up and data collection. The research reported in this publication was supported by funding provided by the CSIR-Crops Research Institute Tissue Culture Laboratory internal generated funds.

Funding Details

The research reported in this publication was supported by funding provided by the CSIR-Crops Research Institute Tissue Culture Laboratory internal generated funds.

References

1. Singh HP, Uma S, Selvarajan R, et al. Micropropagation for production of quality banana planting material in Asia-Pacific. Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB), New Delhi, India. 2011; 92.
2. Hapsari L, Lestari DA. Fruit characteristic and nutrient values of four Indonesian banana cultivars (*Musa spp*.) at different genomic groups. Agrivita. 2016; 38: 303-311.
3. https://www.fao.org/faostat/en/#data/QCL/visualize.
4. https://www.fao.org/3/a0750e/a0750e00.html.
5. Weyori AE, Amare M, Garming H, et al. Agricultural innovation systems and farm technology adoption: findings from a study of the Ghanaian plantain sector. The Journal of Agricultural Education and Extension. 2018; 24: 65-87.
6. Olumba CC, Onunka CN. Banana and plantain in West Africa: Production and marketing. African Journal of Food, Agriculture, Nutrition and Development. 2020; 20: 15474-15489.
7. Dagnew A, Shibru S, Debebe A, et al. Micropropagation of banana varieties (Musa spp.) using shoot-tip culture. Ethiopian Journal of Agricultural Sciences. 2012; 22: 14-25.
8. Alvarado-Ortiz AN, Díaz M, Almodovar W, et al. Crop profile for plantain and banana in Puerto Rico. 2005.
9. Tumuhimbise R, Talengera D. Improved propagation techniques to enhance the productivity of banana (Musa spp.). Open Agriculture. 2018; 3: 138-145.
10. Lal M, Jamwal M, Sood Y, et al. Micropropagation of fruit crops: A review. Plant Sci Today. 2023; 10: 108-117.
11. Uma S, Karthic R, Kalpana S, et al. Evaluation of temporary immersion bioreactors for *in vitro* micropropagation of banana (Musa spp.) and genetic fidelity assessment using flow cytometry and simple-sequence repeat markers. S Afr J Bot. 2023; 157: 553-565.
12. Murashige T. A revised medium for rapid growth and bioassays with tobacco cultures/T. Murashige, F. Skoog. Physiol Plantarum. 1962; 15: 437-497.
13. Doyle J. DNA protocols for plants. Molecular techniques in taxonomy. 1991: 283-293.
14. Christelová P, Valárik M, Hřibová E, et al. A platform for efficient genotyping in Musa using microsatellite markers. AoB Plants. 2011; 2011: plr024.
15. Nei M, Takezaki N. Estimation of genetic distances and phylogenetic trees from DNA analysis. Proc 5th World Cong Genet Appl Livstock Prod. 1983; 21: 405-412.
16. Abdalla N, El-Ramady H, Seliem MK, et al. An academic and technical overview on plant micropropagation challenges. Horticulturae. 2022; 8: 677.
17. García-Gonzáles R, Quiroz K, Carrasco B, et al. Plant tissue culture: Current status, opportunities and challenges. International Journal of Agriculture and Natural Resources. 2010; 37: 5-30.
18. Conde F, Carmona-Martin E, Hormaza JI, et al. *In vitro* establishment and micropropagation of mango (Mangifera indica L.) from cotyledonary nodes. *In Vitro* Cellular & Developmental Biology-Plant. 2023: 1-12.
19. Corozo Quiñonez L, Macías Ponce F, Del Valle Moreira M, et al. Effect of Auxins, Cytokinin and Activated Charcoal on *In Vitro* Propagation of Plantains Barraganete and Curare (Musa AAB). Proc Natl Acad Sci India Sect B - Biol Sci. 2021; 91: 431-440.
20. Kanwar K, Devi V, Sharma S, et al. Effect of physiological age and growth regulators on micropropagation of Aloe vera followed by genetic stability assessment. National Academy Science Letters. 2015; 38: 29-35.
21. Yavuz HB, Çömlekçioğlu N. Effects of Donor Plant Age and Explant Types on Asparagus (*Asparagus officinalis L.*) Micropropagation. International Journal of Agriculture Forestry and Life Sciences. 2022; 6: 12-17.

22. Gaba VP. Plant growth regulators in plant tissue culture and development. Plant development and biotechnology. 2005: 87-99.

23. Noyer J, Causse S, Tomekpé K, et al. A new image of plantain diversity assessed by SSR, AFLP and MSAP markers. Genetica. 2005; 124: 61.

24. Quain MD, Agyeman A, Dzomeku BM. Assessment of plantain (*Musa sapientum L.*) accessions genotypic groups relatedness using simple sequence repeats markers. African Journal of Biotechnology. 2018; 17: 541-551.

25. Infante D, González G, Peraza-Echeverría L, et al. Asexual genetic variability in Agave fourcroydes. Plant Science. 2003; 164: 223-230.

26. Newbury HJ, Howell EC, Crouch JH, et al. Natural and culture-induced genetic variation in plantains (*Musa spp*. AAB group). Aust J Bot. 2000; 48: 493-500.

27. Sales EK, Butardo NG. Molecular analysis of somaclonal variation in tissue culture derived bananas using MSAP and SSR markers. International Journal of Biotechnology and Bioengineering. 2014; 8: 615-622.

28. Bredmose N, Hansen J. Topophysis affects the Potential of axillary bud growth, fresh biomass accumulation and specific fresh weight in single-stem roses (*Rosa hybrida L.*). Annals of Botany. 1996; 78: 215-222.

29. Liu J, Zhang Z, Li Y, et al. Effects of the vegetative propagation method on juvenility in Robinia pseudoacacia L. Forestry Research. 2022; 2.

30. Mudge KW, Brennan EB. 8 Clonal Propagation. Agroforestry in Sustainable Agricultural Systems. 1998; 165: 157.