ACTA
BOTANICA *PLANTAE*

Acta Botanica Plantae : 01–09 (2022)
http://www.actabotanica.org/

ORGINAL RESEARCH ARTICLE

Protocol optimization for Micropropagation of Banana Varieties (*Musa* spp.) Using Shoot-Tip Culture

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Received 25 March 2022 | Revised 29 July 2022 | Accepted 30 August 2022 | Available Online September 02 2022

Citation: Gerema Amente, Megersa Gemechu and Imiru Chimdessa. Protocol optimization for Micropropagation of Banana Varieties(Musa spp.) Using Shoot-Tip Culture. Acta Botanica *Plantae*. V01i02, 01-09. DOI: http://dx.doi.org/10.5281/zenodo.7262848

ABSTRACT

Conventional vegetative propagation of bananas generally has low production, the transmission of diseases, and poor preservationof original plant genetic material. Micropropagation is currently the only practical means of achieving rapid, large-scale production of disease-free quality planting material. The present study was conducted to optimize quick and reproducible in vitro micropropagation protocol for three banana varieties (Grandnaine, Poyo, and Butuza) grown in Oromia Experiments on shoot tip culture initiation, shoot multiplication and in vitro rooting were laid out in completely randomized design with 3x2x2, 5x4x3, and 4x3 factorial treatment arrangements respectively. Data were subjected to analysis of variance (ANOVA) and significant means were separated using Duncan's multiple range tests. About shoot multiplication, Grandnaine showed a maximum of 12.67shoots per explant with a 2.17cm shoot length on a medium fortified with 3.5mg/l BAP alone, while Poyo produced a maximum of 13.00shoots per explant with 2.15cm mean shoot length on a medium supplied with 3.0 mg/lBAP and 0.2mg/l IBA. Likewise, Butuza produced a maximum of 11.33 shootsper explant with a mean shoot length 2.9 cm on medium fortified with 4.0 mg/l BAP + 0.3 mg/l IBA. Half MS -semi-solid medium containing 1.0 mg/l IBA induced the highest rooting with 8 mean root numbers per shoot for Grandnaine. For Poyo, half MS medium supplemented with 1.5mg/l IBA induced the highest rooting response with mean root number per shoot of 7.6. Similarly, ½ MS medium supplemented with 2.0 mg/l IBA induced the highest rooting response with 8 mean root numbers per shoot. In conclusion, this study can be used for rapid and massin Vitro propagation of these three elite Banana varieties.

Keywords: *Micropropagation, Banana Varieties, 6-Benzylaminopurine, acid, Indole butyric acid*

INTRODUCTION

Banana (*Musa* spp. AAA) is an important fruit crop of the Musaceae family, widely grown in developing countries, and is the second largest fruit crop in the world after citrus [10] Banana originated from the South East Asian region, where the greatest diversity of edible bananas are found [16]. Ethiopia is among the tropical countries where its vast areas are suitable for banana cultivation and has also the opportunity for exporting fresh banana fruits. Banana production in the country ranges from homesteads to large commercial plantations under rainfed and/or with supplementary irrigation conditions [3] The materials used for conventional propagation are corms, suckers, and sword suckers. Since on average only 5 to 10 suckers can be obtained per plant per year, the traditional clonal propagation method appears to be unable to supply the increasing demand for healthy planting materials of banana. Conventional vegetative multiplication of bananas has been found to express several negative impacts including, low

production, the transmission of diseases, and poor preservation of original plant genetic material [12]. To augment conventional propagation and to avoid constraints imposed by some pathogens, *in vitro* approach has been considered due to its potential to provide genetically uniform, pest- and disease-free planting materials . Propagation of bananas through in Vitro techniques has been reported by several authors using different explant sources as well as regeneration pathways [2] [8], [13] [7]. Shoot tip culture has been routinely used for the rapid clonal propagation of banana genetic resources since 1985. Micro propagated plants establish more quickly, grow more vigorously, have a shorter and more uniform production cycle, and produce higher yields than conventional propagules [15] Even though many reports are available on banana micropropagation through shoot tip culture, plants could exhibit great variation under *in vitro* conditions in terms of shoot establishment, shoot proliferation, and regeneration of shoots and roots because of several factors such as genotype, explant type, culture media composition, plant growth regulators (PGR) and culture environment [18] [20].

Therefore, this study was started to optimize quick and reproducible *in vitro* micropropagation protocol for three banana varieties grown in Ethiopia. The protocols mainly the composition of media that include concentrations and combinations of plant growth regulators are largely plant genotype-dependent and are also influenced by water quality and techniques used in different laboratories. For this purpose, the effects of different cytokinins and auxins at various concentrations on shoot initiation, multiplication and elongation, and *in vitro* rooting were studied. The effect of an acclimatization media mix on banana plantlets growth was also studied.

MATERIALS AND METHODS

Plant Materials

Three cultivated banana varieties in Ethiopia, namely Poyo, Grand naine andButuza were used as experimental materials. Sword explants were obtained from healthy-looking, field-grown varieties from Adami Tulu Agricultural Research Center (ATARC) banana propagation nursery. The pseudo stems at the lower parts of the suckers containing meristems were used as explants. The shoot tips were the starting materials. This study was conducted at the Plant Tissue CultureLaboratory of ATARC, Oromia, Ethiopia.

Culture Media Preparation and Sterilization

The culture medium used for this study was a modified Murashige and Skoog (MS, 1962) basal medium. The required amount of macronutrients, micronutrients, and vitamins from respective stock solutions were dissolved in double distilled water along with 30 g/L sucrose and 7g/l agar-agar type-I. The final volume was made up to the required level with double distilled water and then divided into the required volume of treatments, to which amount of PGRs from stock solution was added in combinations at different concentrations. The pH was adjusted in all cases to 5.8 by using 1N NaOH and/or 1N HCL before autoclave and gelling with agar. The media was poured into washed and dried culture jars of 60 ml volume and then, capped and labeled properly. The medium was autoclaved at 1.2 KPa and 121^{o}C for 20 min and then cooled at room temperature before use. The autoclaved medium was kept on a shelf for three days (72hr) to make sure that there is no microbial contamination

Explant Preparation and Surface Sterilization

Explants were excised from young suckers of the three banana varieties. The superfluous corm tissue, roots, and leaf sheathes were trimmed and removed by a sharp knife. Explants were washed carefully in running tap water for 15min with a detergent solution (largo) to remove adherent soils. The leaf sheaths near the bases were again removed leaving the young leaves around the meristem until the shoot tip became about 1.5 cm in length. Then the explants were dipped in a 3gm/l solution of mancozeb fungicide for 10min and rinsed three times with autoclaved distilled water. The explants were then briefly sterilized with 2% sodium hypochlorite solution for 15min followed 70% ethanol for one minute. After rinsing three times with autoclaved distilled water, the explants were excised into final size (about 5 mm) under a laminar air flow hood

Shoot Tip Culture Establishment

Surface sterilized explants were placed on an MS medium containing a combination of N6 benzyl aminopurine (BAP: 1.5 and 2.5 mg/l) and indole-3-acetic acid (IAA: 0.0 and 0.5 mg/l) for shoot tip initiation. The factorial treatment was 3x2x2 combination arrangements. Thirty culture jars with

DOI: http://dx.doi.org/10.5281/zenodo.7262848

each jar having one shoot tip explant were cultured for each of the three varieties (30 shoot tips per treatment). The initiated cultures were incubated for 8 weeks aseptically at 25±2°C under 16/8hr cool white, fluorescent lights. Data of shoot initiation were recorded four weeks after culture.

In vitro shoot proliferation

The same MS medium supplemented with a combination of BAP (2.0, 2.5 3.0, 3.5, and 4.0 mg/l) and IBA (0.0, 0.2, 0.3, and 0.5 mg/l) was used for shoot proliferation. The experiment was laid out inCRD with three factorial combinations of five levels of BAP; four levels of IBA and three levels of banana varieties (Poyo, Grandnaine, and Butuza) resulting in 5x4x3 factorial treatment structure. After 8 weeks of the initiation stage contamination-free shoots were decapitated and split longitudinally into two or more parts depending on vigor and thickness. Then, they were transferred to a multiplication medium. However, smaller shoots were not split; rather they were put three together in a culture vessel. The multiplication rate was studied by subculturing the shoots on MS media every 3 weeks for 5 cycles. Data on the number of shoots per explants, the number of leaves, and the length of shoots were carefully recorded during each sub culturing.

In Vitro Rooting of Shoots

In the root development stage, well-grown shoots with expanded leaves were separated and transferred singly to fresh rooting half MS medium with different concentrations of indole butyric acid (IBA) (0.5, 1.0, 1.5, and 2.0 mg/l). After 6 weeks of culture, and root growth,data on the number of roots per shoot, and length of roots (cm) were recorded. For each treatment, fourshoots in each jar (12 shoots per treatment) were lined up randomly in CRD with three replications.

Acclimatization and Hardening of Plantlets

In vitro rooted plantlets were taken out of the culture jars and the roots were carefully washed with water to remove all the traces of the rooting medium. Plantlets were disinfected with Ridomil (2.0 g/l) for 3min to prevent fungal infections before transplanting. Individual plantlets were then transferred into small polybags filled with sterile soil mixtures top forest soil, farm yard manure, and sand soil in 2:1:1/2 ratio. The soil mixtures were sterilized by drying oven at 180°C for an hour. Then, plants were later transferred to bigger polybags that were filled with top forest soil and manure in a ratio of 2:1. The hardened plants were finally transferred to the field and successfully established and evaluated for it fruit.

Experimental Design and Data Collection

The treatments were arranged in a Completely Randomized Design (CRD) with factorial arrangements. In the initiation stage, the number of explants that survived and initiated shoots was recorded was expressed as a percentage. Explants were subcultured five times and after each subculture, the number of shoots per explant, number of leaves per shoot and shoot length for each Variety were counted. In the rooting stage, leaf numbers (determined by counting all leaves per plant), and root numbers (determined by counting all roots per plant), were examined. In the acclimatization and hardening stage, the survival of transplanted plants (determined by counting all live plants), plant height, pseudo stem diameter, and leaf numbers was also examined.

Data Analysis

In the present study, the treatments were the plant growth regulators (the cytokinin - BAP; and the auxins - IBA) with various concentration levels and the banana varieties at each micropropagation stage. Furthermore, survival percentages were calculated for acclimatization and hardening plantlets.. Experimental data were analyzed By R software using Multiple Analyses of Variance at 95% of confidence level. When F-Test showed statistical significance at $p < 0.05$ level, means were separated according to Duncan Multiple Range Test (DMRT) Procedure.

RESULTS AND DISCUSSION

Shoot Initiation

In the present study, *in vitro* culture of banana shoot tips resulted in hard meristematic ball-like structure in initiation media containing different concentrations of BAP and IBA. The cultured shoot tip turned brown in color from the initial creamy white a few days after inoculation. Four weeks later, the external leaf primordia of explants turned green and a globular hard coat mass grew from which adventitious plantlets were developed. Among the treatment combinations considered, the maximum

Table 1: Effect of different concentrations of BAP along with IBA and BAP alone on shoot initiation of three banana varieties

Treatments PGR(mg/l)	Name of banana varieties								
	Grandnaine			*Poyo*			*Butuza*		
	explants cultured	initiated shoots	survival %	explants cultured	initiated shoots	survival %	explants cultured	initiated shoots	survival %
2BAP+0.0IBA	20	10	50	20	16	80	**30**	**28**	**93.3**
2BAP+0.5IBA	20	15	75	20	16	80	30	26	86.6
2.5BAP+0.0IBA	20	17	85	**20**	**18**	**90**	30	24	76.6
2.5BAP+0.5IBA	20	18	90	20	17	85	30	21	70
3BAP+0.0IBA	**20**	**20**	**100**	20	16	80	30	22	73.3
3.5BAP+0.5IBA	20	17	85	20	14	70	30	20	66.6

shoot tip initiation response of 100% sprout was obtained from explants cultured on MS medium supplemented with 3.0mg/l BAP the *Grandnaine* banana variety. Likewise, poyo and Butuza varieties gave maximum shoot imitation on MS medium supplemented with 2.5mg/l BAP and 2.5mg/l BAP respectively (Table 1). A similar result has been reported by replace references by number system like [1] [2] etcusing MS medium supplemented with 2.0 to 3.0 mg/l BAP. Cronauer and Krikorian (1984) and Vuylsteke (1998) also reported BAP as the most commonly preferred cytokinin used in banana tissue culture. Furthermore, Al-Amin *et al.* (2009) observed the color change of culture meristems to brown in 4 to 5 days and development of a green hard ball-like structure after 30 to 35 days of inoculation. Azam *et al.* 2009 found that cultured shoot tips were visible as a swelling and greenish color after 10–15 days of inoculation in MS media supplemented with different concentrations of BAP. Al-amin *et al.* 2010 observed a meristematic ball like structure in regeneration media containing different concentrations of BAP and NAA.

Invitro Shoot Multiplication

After 8 weeks of culture initiation, when shoots with at least one leaf emerged, shoots were transferred to multiplication medium (Figure 4). It has been reported that multiple shoots could be produced from sliced shoot tips of bananas and plantain [5]). Analysis of variance revealed that the interaction effects of Variety, BAP, and IBAwas significant (Variety * BAP * IBA = $p < 0.05$) on the number ofshoots per explant, average shoot length (cm), and the number of leaves per shoot. The interaction of genotype, BAP, and IBA indicated that all three factors are dependent on each other for *in vitro* shoot proliferation of banana varieties. The maximum number of shoot per explants were obtained on MS medium supplemented with a combination of BAP and IBA at concentrations of 3.5/0.0, 3.0/0.2, and 3.5/0.3 mg/l for *Grand Naine*, *Poyo* and*Butuza* respectively(Table-3, figure 4,5 and 6). Adenine-based cytokinin particularly BAP is the most commonly preferred cytokinin to affect shoot multiplication rate in several *Musa* spp. [11]; [18] [23] stated the highest shoot number per explant (3.4) with 5.0 mg/L BAP+2.0 mg/L IBA. The formation of multiple shoots and buds in banana varieties were promoted by supplementing the reported with relatively high concentrations of cytokinins. With the increase in BAP concentration from 2mg/l to 3.5mg/l numbers of shoots/ explant increased were increased from 4.67 to 12.67, 5.0 to 7.3 and 6.67 to 8.0 in*Grand Naine*, *Poyo* and*Butuza* respectively. The number ofshoots increased with the increase of BAPconcentration up to 3.5 mg/l and then decreased. [6] reported an increase of shoots with an increase of BAP hormone concentration up to 5mg/l and the decrease. It was experimental that all the explants of banana varieties did not behave similarly *in vitro* in terms of multiplication.

Table 2: ANOVA summary of the Effect of BAP and IBA on invitro Shoot Multiplication

Source of variation	DF	Mean Square (MS)		
		No of shoots	Shoot length(cm)	No of leaves
IBA	3	30.272*	0.9688*	2.4667 *
BAP	4	29.778**	1.8958**	10.2417*
Varieties	2	5.272*	4.0112*	10.4222 *
IBA*BAP	12	22.041**	0.5957*	2.8231**
IBA* Varieties	6	38.694**	1.4322**	5.9333 **

DOI: http://dx.doi.org/10.5281/zenodo.7262848

Continued..

BAP* Varieties	8	15.015***	0.5073***	3.5750***
IBA*BAP* Varieties	24	23.012***	0.5089***	2.5676***
CV%	26.6	22.6	18.2	
SE	2.713	0.6898	1.44	

DF =Degree of freedom, SE=Standard Error, BAP = 6- Benzylaminopurine, IBA = Indole butyric acid,CV = Coefficient of variation, *=p≤0.05, **=p≤0.01,***=p≤0.001

Figure 1: Grand naine shoot initiation on 3mg/l BAP

Table 3: The effect of BAP and BAP on number of shoots per explant, Shoot length and number of leaves per shoot

PGR(mg/l)		Banana Varieties								
		G/Naine			Poyo			Butuza		
BAP	IBA	NS	SL	NL	NS	SL	NL	NS	SL	NL
2.0	0.0	4.67	2.10	3.00	5.00	1.42	3.00	6.67	1.83	1.67
2.5		5.67	1.50	4.00	5.67	1.53	2.33	6.33	2.13	3.33
3.0		6.67	3.42	3.67	6.00	2.03	4.33	8.00	1.73	2.33
3.5		**12.67**	2.17	3.00	7.33	2.28	2.33	6.00	2.53	3.33
4.0		8.00	1.25	2.33	7.67	2.02	4.33	7.33	2.17	4.33
2.0	0.2	7.00	2.17	3.67	7.33	2.70	2.00	7.00	1.40	3.67
2.5		6.67	1.67	4.33	8.00	1.79	2.00	6.33	2.30	5.33
3.0		5.67	1.13	3.00	**13.00**	2.15	3.33	9.00	2.02	4.33
3.5		6.00	1.20	4.33	8.67	2.65	2.33	7.00	1.60	5.67
4.0		5.00	2.00	2.67	6.00	2.47	3.67	6.00	2.38	5.00
2.0	0.3	5.33	1.77	1.67	7.67	2.90	3.00	5.00	1.76	2.67
2.5		7.00	1.23	3.00	7.67	2.35	3.33	7.67	1.99	5.00
3.0		5.67	1.37	2.00	8.33	2.50	2.33	9.00	2.77	3.33
3.5		5.00	2.03	4.67	6.67	2.62	4.00	**11.33**	2.98	6.00
4.0		6.00	2.47	3.67	5.67	2.83	2.00	8.00	2.36	2.00
2.0	0.5	5.67	2.57	3.00	6.00	2.50	3.33	5.67	1.53	2.67
2.5		6.00	1.77	3.00	7.00	2.76	1.67	4.67	1.40	2.67
3.0		7.33	1.80	4.00	5.33	1.94	1.33	5.00	1.61	4.00
3.5		7.00	2.23	6.00	4.67	3.13	3.33	4.67	2.42	4.00
4.0		4.33	2.00	5.00	7.67	3.33	3.67	6.33	2.30	2.67
CV%		16.6	12.6	8.2	16.6	12.6	8.2	16.6	12.6	8.2
SE		2.71	0.69	1.44	2.71	0.69	1.44	2.71	0.69	1.44

Ns=number of shoots, SL=Shoot length, NL=number of leaves

In Vitro Rooting of Shoots

ANOVA showed a significant (p≤0.05) effect of all main and interaction effects of varieties and, IBA on the number of roots per shoot, number of leaves per shoot, and shoot length in three Varieties indicating the interdependence of these factors *in vitro* root induction. Fine roots began to be induced from the basal portion of the shoots after 9-11 days in *Grandnaine*, while 10-13 days in *Poyo* and *Butuza* cultivars on treatments fortified with different concentrations. Rooting can be stimulated when individual shoots are transferred to a basal medium without any PGR [15], [18]. However, auxins induce further root

Figure 2: Poyo shoot initiation on 2.5mg/l BAP

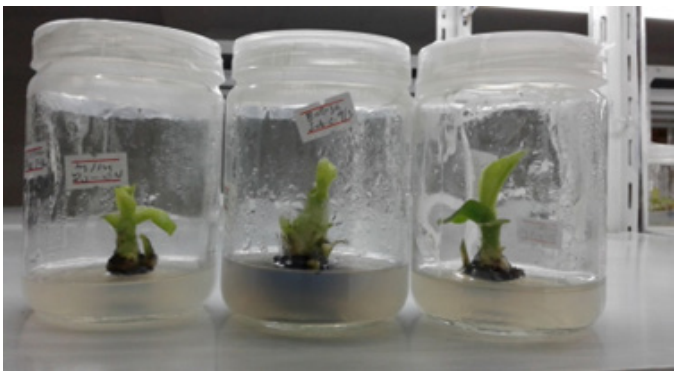

Figure 3: Butuza Shoot Initiation on 2mg/l BAP

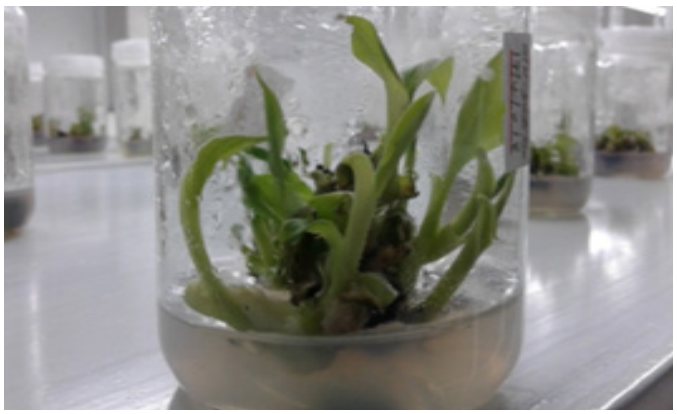

Figure 4: Grand naine shoot proliferation on 3.5mg/l BAP

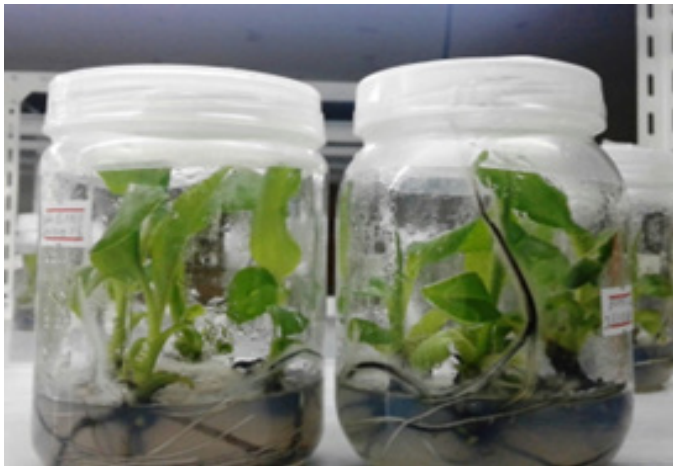

Figure 5 Poyo shoot multiplication on 3mg/l BAP and 0.2mg/l IBA

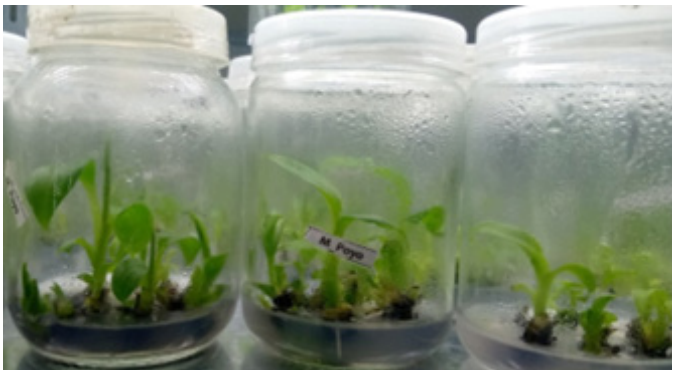

Figure 6 : Butuza Shoot multiplication on 3mg/l BAP and 0.3mg/lIBA

initiation in bananas [18].

The highest numbers of roots (8.0, 7.67, and 8.0) were observed on the medium supplemented with 1mg/l IBA, 1.5mg/l IBA, and 2mg/l IBA, for *Grandnaine, Poyo,* and *Butuza* respectively. While in the control medium produced 4.33, 3.67, and 4.0 roots per shoot for *Grandnaine*, *Poyo* and *Butuza* respectively. These results are in agreement with the findings of **[14]** who obtained 11 maximum numbers of roots per shoot on 1mg/l IBA for red banana variety. Similarly, **[9]** reported a maximum number of roots (7.80) in BARI Kola-4 on ½MS medium supplemented with 0.5 mg/l IBA. IBA is known to plays an important role in the formation and development of rooting. Root formation and plant regeneration with IBA has been reported by [1] and [11].

Figure 7: Grand naine rooting on 1mg/l IBA

Acclimatization of three banana varieties

After the sufficient shoot and root development, the small plantlets were taken out from the culture vessel carefully without damaging any roots. Excess media around the root was washed off by running tap water to prevent further microbial infection. *In vitro* rooted plantlets were transferred into a plastic tray filled with sterile soil mixtures top forest soil, farm yard manure, and sand soil in 2:1:1/2 ratio. Then, plants were later

DOI: http://dx.doi.org/10.5281/zenodo.7262848

Figure 8: Poyo shoot rooting on 1.5mg/l IBA

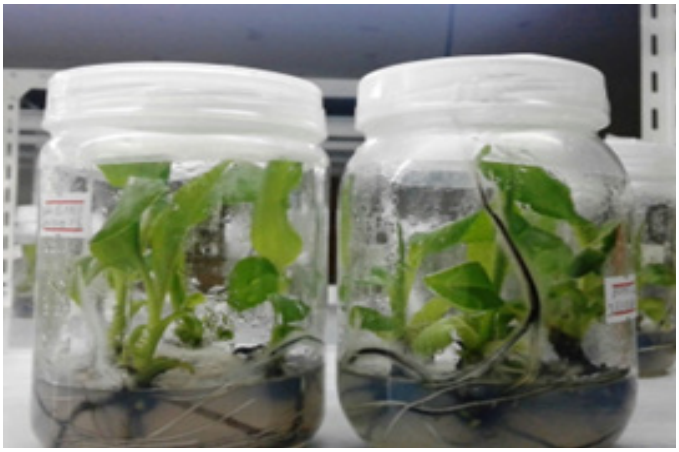

Figure 9: Butuza shoot rooting on 2mg/l IBA

Table 4: Effect of IBA different concentration on in vitro rooting and root growth on half MS media

IBA	Name of banana Varieties								
	Grandnaine			*Poyo*			*Butuza*		
	NR	NL	RL	NR	NL	RL	NR	NL	RL
0.0	4.33	4.67	4.00	3.67	4.00	3.67	4.00	3.00	3.00
0.5	6.00	2.33	3.00	5.67	5.00	4.33	5.00	4.67	4.00
1.0	8.00	5.00	5.33	4.67	4.67	5.67	5.33	5.33	5.67
1.5	5.00	3.67	4.67	7.67	5.67	6.33	6.33	4.67	4.67
2.0	6.67	4.00	3.67	6.00	3.33	3.33	8.00	7.33	3.67

Banana Varieties								
Grand naine			**Poyo**			**Butuza**		
No plant lets potted	No of plantlets survived	% of survival	No plant lets pot-ted	No of plantlets survived	% of survival	No plant lets pot ted	No of plantlets survived	% of survival
300	270	**90%**	250	230	**92%**	270	250	**92.5%**

transferred to bigger polybags containing top forest soil and manure in a ratio of 2:1. The hardened plants were finally transferred to the field and successfully established for further evaluation.

CONCLUSION

From the result obtained in the present study, it is concluded that the developed protocol is helpful for rapid *in vitro* propagation of the banana planting materials and hence enhances the availability of healthy and true-to-type planting materials accordingly, the information below was obtained For shoot tip culture initiation and establishment Grand nainePoyo and Butuza varieties showed best performance on 3.0 mg/L BAP, 2.5 mg/L BAP and 2.0 mg/L BAP respectively, without IBA concentration A combination of 3.0mg/LBAP + 0.2mg/LIBA was the best combination forshoot multiplication of Poyowhile **3.5mg/l BAP** without IBA and 4.0mg/L BAP + 0.3 mg/l IBAwere the most selected combinations for Grand naineand Butuza respectively.

For *in vitro* rooting, half-strength MS semi-solid medium fortified with 1.0 mg/l IBA alone was the best concentration for genotype Grandnaine, while half MS medium supplemented with 1.5 mg/L and 2.0mg/lIBA were the best growth regulator concentration for poyo and Butuza respectively.

Recommendations

Based on the results of the present study, the following recommendations were made In the future, it will be better to determine the optimum volume of liquid medium per a given jar or flasks to develop efficient protocols for the above-mentioned varieties using a Bioreactor system. It is also recommended to optimizeprotocolsforthesevarietiesusinganothertype

of plant growth hormone concentration and combination to get the best multiplication. It is best to use an Automatic greenhouse for the primary acclimatization of banana plantlets under strictly controlled Relative humidity and temperature.

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