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

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Abstract

A study was carried out at the Tissue Culture Laboratory of Melkassa Agricultural Research Centre, Ethiopian Institute of Agricultural Research (EIAR) to investigate the effects of different types and concentrations of cytokinins and auxins on shoot initiation and multiplication, and in vitro shoot rooting of three banana varieties using shoot-tip explants. Shoot initiation was greater on Murashige and Skoog (MS) basal medium supplemented with 3 mg/l N6-benzylaminopurine (BAP) for Dwarf and Giant Cavendish while 2 mg/l for Poyo varieties. Among the different concentrations of plant growth regulators (PGR) tested, MS medium supplemented with combinations of BAP and indole-3-acetic acid (IAA) at 3+0.4, 4+0.4 and 3+0.2 mg/l for Dwarf, Giant and Poyo respectively, were best combinations for high rates of shoot proliferation and elongation. Further multiplication of shoots required up to 5 times subculturing of 1 month each on the same media combination. In this study, about 3-fold multiplication rate was achieved during every subculture. Better rooting was obtained when the shoots were cultured on MS medium with 2.12 mg/l α-naphthaleneacetic acid (NAA) for Dwarf and Giant while 1.74 mg/l indol-3-butyric acid (IBA) for Poyo. In vitro rooted plantlets were transferred to the lathouse for acclimatization and hardening. The best growth was recorded for plantlets transplanted on potting media containing a 3:1 ratio (v/v) of sugarcane filter cake and sand. The hardened plants were transferred and well established to the field.

Key Words: Bananas, *Musa* spp., micropropagation, shoot-tip, plant growth regulators

Introduction

Bananas (including plantains) (*Musa* spp.) are one of the most important fruit crops of the world (Nelson *et al*., 2006). They are giant perennial herbs (2 to 9m), some species of which can grow up to 15m tall. The banana plant is a monocotyledon with a false stem comprising of leaf sheaths and an underground true stem which is able to produce suckers by which the plant can reproduce vegetatively. Each false stem produces a single inflorescence, the female flowers of which give rise, either parthenocarpically or following fertilization, to the banana fruits (Nelson *et al*., 2006; Oselebe *et al*., 2006).

Bananas are originated from Southeast Asia where a region considered as the primary centre of diversification and the earliest domestication has occurred (Stover and Simmonds, 1987). The low land areas of West Africa also contain the largest range of genetic diversity of plantains (*Musa* AAB) (Ortiz and Vuylsteke, 1994). In East Africa, bananas are highly evolved into an important zone of secondary genetic diversity for the East African highland bananas (*Musa* AAA) (Smale, 2006). Nowadays, bananas are grown in more than 100 countries throughout the tropics and subtropics (Frison and Sharrock, 1999; Irish *et al*., 2009), both as a staple food and export commodity (Oselebe *et al*., 2006).

Bananas are produced over an area of approximately 10 million ha worldwide, with an annual production of 88 million tons (Frison and Sharrock, 1999). In terms of gross value of production, bananas are the fourth most important food crop (Frison and Sharrock, 1999; Nelson et al., 2006). The world annual banana production has increased to approximately 105 million metric tons in the 2005 calendar year (INIBAP, 2006). As a staple, bananas contribute to the food security of millions of people in much of the developing world, and when traded in local markets, they provide income and employment to rural populations. Bananas are the most exported fresh fruits of the world in terms of volume and value. According to (FAOSTAT, 2006), total world export for bananas was over 15.9 million tons in 2006. In Africa, bananas provided more than a quarter of the carbohydrate requirements for over 70 million people (IITA, 1998). Eastern and Southern Africa produced over 20 million tons of bananas which accounted for 25.58% of total world output (Karamura *et.al*., 1999).

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 homestead to large commercial plantations under rainfed and/or with supplementary irrigation conditions (Seifu, 1999). At present, bananas are the leading fruit crop produced in the country both in terms of area coverage and production where the bulk is produced in traditional agricultural system. According to CSA (2009), among fruit crops produced by peasant holdings during the main season of 2008/9, banana cultivation covered 29,064 ha (60.56% acreage) and contributed 194,333 tons of the production (55.3%).

Most commercial bananas are propagated vegetatively due to the high degree of sterility and polyploidy of the edible varieties (Stover and Simmonds 1987). 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. The materials used for conventional propagation are corms, and small and large suckers (Cronauer and Krikorian, 1984; Arias, 1992). However, these conventional materials are not the ideal propagule, because they often carry weevils or borers, fungal pathogens, nematodes, and viruses (Arias, 1992) and also suffer from slow multiplication, bulkiness, and poor phytosanitary quality (Vuylsteke, 1998). In order to augment conventional propagation and to avoid constraints imposed by some pathogens, *in vitro* approach has been considered (Tripathi 2003). Several researchers have reported the regeneration of banana genotypes via *in vitro* micropropagation (Cronauer and Krikorian, 1984; Kagera *et al*., 2004; Madhulatha *et al*., 2004). Shoot tip culture has been routinely used for the rapid clonal propagation of banana genetic resources since 1985. As compared to conventional propagation, *in vitro* micropropagation provides higher rates of multiplication, produces clean planting material, and requires small space to multiply large number of plants (Arias, 1992; Vuylsteke, 1998). Micropropagated plants establish more quickly, grow more vigorously, have a shorter and more uniform production cycle, and produce higher yields than conventional propagules (Robinson *et al*., 1993).

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 (Vuylsteke, 1998). Therefore, this study was initiated to optimize rapid and reproducible *in vitro* micropropagation protocol for three banana varieties grown in Ethiopia. For this purpose, the influences of different cytokinins and auxins at various concentrations on shoot initiation, multiplication and elongation, and *in vitro* shoots rooting were investigated. The effect of acclimatization media mix on banana plantlets growth was also studied.

Materials and Methods

Plant Materials

Three widely cultivated Cavendish group banana varieties in Ethiopia, namely Dwarf Cavendish, Giant Cavendish and 'Poyo' were used as experimental materials in this study. The explants were obtained from healthy looking, field grown sword suckers of these varieties from Melkassa Agricultural Research Center (MARC) banana propagation nursery. The pseudostems at the lower parts of the suckers containing meristems were used as explants. The shoot tips (meristem and a few leaf primordia) were the starting materials. This study was conducted at the Tissue Culture Laboratory of MARC, Ethiopia.

Explant Preparation and Surface Sterilization

Explants were excised from young suckers (0.75 to 1.0 m) of the three banana varieties. The superfluous corm tissue, roots, and leaf sheathes were trimmed and removed from the pseudostem by sharp knife. Explants were washed thoroughly in running tap water for 15-20 min with detergent solution to remove adherent soils. The leaf sheaths near the bases were again removed from the pseudostem leaving the young leaves around the meristem until the shoot tip became about 2 cm in length. The explants were then briefly rinsed with 70% ethanol, followed by 2% sodium hypochlorite solution for 10 min. After rinsing three times with sterile distilled water, the explants were excised into final size (about 5 mm) under aseptic conditions.

Shoot Tip Culture Establishment

The culture medium used for this study was modified Murashige and Skoog (MS, 1962) basal medium containing 30 g/l sucrose and gelled with 8 g/l of agar. The pH of the medium was adjusted to 5.7 using NaOH or HCl (0.1 or 1N) before autoclaving. PGRs were also added to the medium prior to sterilization. The medium was autoclaved at 1.2 KPa and 121ºC for 20 min, and then cooled at room temperature before use.

Surface sterilized explants were placed on MS medium containing a combination of N6-benzylaminopurine (BAP: 2.0 and 3.0 mg/l) and indole-3-acetic acid (IAA: 0.0 and 0.65 mg/l) for shoot tip initiation. The initiated cultures were incubated for 8 weeks aseptically at 25±2ºC under 16 h cool white, fluorescent lights.

Evaluation of Multiplication Rate

The same MS medium supplemented with a combination of BAP (3.0 and 4.0 mg/l) and IAA (0.0, 0.2, 0.4, and 0.6 mg/l) was used for shoot proliferation. After 8 weeks of initiation stage contamination-free shoots were decapitated at 7 to 10 mm height and split longitudinally into two or more parts depending on vigor and thickness. Then, they were transferred to multiplication medium. However, smaller shoots were not split; rather they were put two or three together in a culture vessel. The multiplication rate was studied by subculturing the shoots on MS media every 3 weeks for 7 cycles.

In Vitro Rooting of Shoots

In root development stage, well grown shoots with expanded leaves were separated and transferred singly to fresh rooting MS medium with different concentrations of α-naphthalene acetic acid (NAA) (0.53, 1.06, 1.59, and 2.12 mg/l), IAA (0.5, 1.0, 1.5, and 2.0 mg/l), or indolbutyric acid (IBA) (0.58, 1.16, 1.74, and 2.32 mg/l). Root growth and development was assessed 6 weeks after shoots were transferred to rooting medium.

Acclimatization and Hardening of Plantlets

Elongated and rooted plantlets (about 6 cm with 3 to 4 leaves) were taken out from culture vessels and roots were carefully washed thoroughly with running tap water to remove the agar. Plantlets were disinfected with Benomyl or Ridomyl (2.0 g/l) for 3

min to prevent fungal infections before transplanting. Individual plantlets were then transferred into small polybags filled with various sterile potting mixtures (sand to soils or sugarcane filter cake) in a 1:3 ratios (v/v). The potting mix was fumigated with 5% formalin ten days prior to planting (kept covered for 3 days and then aerated without cover for remaining 7 days). Plants were kept inside chambers covered with transparent plastic for a week in the lathouse to maintain high humidity (80 to 90%) for acclimatization and hardening. The humidity was gradually reduced and plantlets were kept outside the chamber. Then, plants were later transferred to bigger polybags that were filled with forest soil, sand and manure in the ratio of 2:1:1. The hardened plants were eventually transferred to the field and successfully established for further evaluation.

Experimental Design and Data Collection

The treatments were arranged in a Completely Randomized Design (CRD) with factorial arrangements. Each treatment consisted of twenty-one explants in three replications.

In the initiation stage, the number of explants survived and sprouted was recorded which was expressed as percentage. Explants were subcultured seven times and after each subculture, the number of shoots per explant for each cultivar was counted and the rate of multiplication for each variety was determined by overall averaging of the mean number of shoots per explant in each of the five successive subcultures over a period of about 5 months. In the rooting stage, plant height (determined by measuring the height between the starting point of the pseudostem and the point the first leaf emerged), pseudostem diameter (determined by measuring the distance above 1 cm of pseudostem), leaf numbers (determined by counting all leaves per plant), root numbers (determined by counting all roots per plant), and average root length (determined by measuring 3 randomly chosen roots) were examined. In the acclimatization and hardening stage, survival of transplanted plants (determined by counting all live plants), plant height, pseudostem diameter, and leaf numbers were also examined.

Data Analysis

In the present study, the treatments were the plant growth regulators (the cytokinin - BAP; and the auxins - IAA, IBA and NAA) with various concentration levels, and the banana varieties at each micropropagation stage. Furthermore, the different potting mixtures were considered for acclimatization and hardening experiment.

Experimental data were analysed using Multiple Analysis 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 hard meristematic ball like structure in initiation media containing different concentrations of BAP and IAA. The cultured shoot tip turned brown in color from the initial creamy white in a few days after inoculation. Four weeks later, the external leaf primordia of explants turned green and globular hard coat mass grew from which adventitious plantlets were developed (Figure 1A). Among the treatment combinations considered, the maximum shoot tip initiation response (complete survival and 90 to 100% sprout) was obtained from explants cultured on MS medium supplemented with 2.0 to 3.0 mg/l BAP alone for the three banana varieties (Table 1). A similar result has been reported by Muhammad *et al*. (2004) using 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 a development of a green hard ball like structure after 30 to 35 days of inoculation.

Table 1. Effect of different concentrations of BAP along with IAA, and BAP alone on shoot initiation of three banana varieties

Treatments (mg/l)	Banana Varieties					
	Dwarf Cavendish		Giant Cavendish		Poyo	
	Survival (%)	Sprout (%)	Survival (%)	Sprout (%)	Survival (%)	Sprout (%)
2 BAP + 0.0 IAA	100	90	90	70	100	90
2 BAP + 0.65 IAA	100	80	90	70	100	70
3 BAP + 0.0 IAA	100	100	100	90	90	60
3 BAP + 0.65 IAA	100	70	90	80	100	90

Shoot Multiplication

After 8 weeks of culture initiation, when shoots with at least one leaf are emerged, sliced or unsliced shoots were transferred on multiplication medium (Figure 1B). It has been reported that multiple shoots could be produced from sliced shoot tips of banana and plantain (Cronauer and Krikorian, 1984). The results of the number of shoots obtained from each propagule and the shoot multiplication rates for the average of five subcultures are given in Table 2. The highest shoot multiplication rate was obtained on MS medium supplemented with a combination of BAP and IAA at concentrations of 3.0/0.4, 4.0/0.4, and 3.0/0.2 mg/l for Dwarf Cavendish, Giant Cavendish and Poyo, respectively. Using the average multiplication rate for each variety, the mean number of shoots per explant obtained after five subcultures for Dwarf Cavendish, Giant Cavendish and Poyo were 263.94, 277.17, and 335.54 in that order. Auxins and cytokinins have been reported as the most frequently used PGRs for banana micropropagation (Vuylsteke, 1998). Adenine-based cytokinin particularly BAP is the most commonly preferred cytokinin to affect shoot multiplication rate in several *Musa* spp. (Cronauer and Krikorian, 1984; Vuylsteke, 1998). Differences in rate of multiplication of different *Musa* genotypes under *in vitro* conditions have also been

reported (Vuylsteke, 1998; Muhammadi *et al*., 2004). In this study, it was observed that the average rate of multiplication was different among the explants of the different genotypes as indicated in Table 2. Consequently, the results showed that Poyo variety was most productive and produced maximum number of shoots followed by Giant Cavendish and Dwarf Cavendish.

In the current study, subculturing of the shoots for multiplication was carried out for 7 cycles. It was observed that the cultures showed higher rate of multiplication for the first five subcultures However, after the 5th cycle the multiplication rate was declined. Subculturing induced multiple axillary shoots where more than a three-fold increase in multiplication was seen by three weeks after the first subculture. Further transfer in the same medium resulted in about 2 to 4 fold increase in proliferation at every subculture cycle.

Table 2. *In vitro* shoot proliferation of banana varieties on media containing different plant growth regulators at various concentrations

Treatments (mg/l)	Banana Varieties					
	Dwarf Cavendish		Giant Cavendish		Poyo	
	No. of shoots/explant	Multi. Rate	No. of shoots/explant	Multi. Rate	No. of shoots/explant	Multi. rate
3 BAP + 0.0 IAA	3.33	2.85	4.19a	3.23abc	5.28	3.42
3 BAP + 0.2 IAA	3.28	3.00	2.76bc	2.47c	3.80	3.33
3 BAP + 0.4 IAA	3.14	3.33	2.09c	2.33c	2.28	3.09
3 BAP + 0.6 IAA	3.04	3.09	3.04abc	3.61ab	3.52	4.14
4 BAP + 0.0 IAA	3.38	3.57	4.33a	3.28abc	3.95	2.80
4 BAP + 0.2 IAA	3.19	3.19	3.95ab	2.42c	3.47	2.71
4 BAP + 0.4 IAA	3.61	3.00	3.76ab	4.19a	2.76	3.04
4 BAP + 0.6 IAA	3.00	2.33	2.61bc	3.14bc	3.23	3.04
Mean	3.25	3.05	3.34	3.08	3.54	3.20
SE±	0.41ns	0.32ns	0.48*	0.34*	0.39ns	0.33ns

Key: multi rate=multiplication rate; SE=standard error; ns=not significant; *=significant
Means followed by the same letters within a column do not differ significantly.

In Vitro Rooting

After every five cycle subculturing, vigorously growing shoots with expanded leaves were excised and cultured separately on fresh rooting medium to encourage shoot elongation and formation of basal roots. The basal tufts of rooting were emerged in all of the transferred shoots after a few weeks (Figure 1C). Rooting can be stimulated when individual shoots are transferred to a basal medium without any PGR (Cronauer and Krikorian, 1984; Jarret *et al*., 1985). However, auxins induce further root initiation in bananas (Vuylsteke, 1989). The rooting ability data are presented in Table 3. After 6 weeks, the best rooting was obtained when the shoots were grown on MS medium with 2.12mg/l NAA for Dwarf Cavendish and Giant Cavendish while 1.74mg/l IBA for Poyo varieties. In the current study, shoots developed roots and were ready for acclimatization in 45 days after they were transferred to elongation and rooting medium. A similar result was reported by Vessey and Rivera (1981) that the root formation was occurred 50 days after shoot transfer. However, Berg and Bustamante (1974) noted that plantlets needed 2 to 3 months for root formation.

Table 3. Effect of various auxins with different concentrations on *in vitro* shoot elongation and rooting of three banana varieties

Treatments (mg/L)			Dwarf Cavendish					Giant Cavendish					Poyo				
IAA	IBA	NAA	PH (cm)	SD (cm)	NL	NR	RL (cm)	PH (cm)	SD (cm)	NL	NR	RL (cm)	PH (cm)	SD (cm)	NL	NR	RL (cm)
0.0	0.0	0.0	3.6d	3.3ab	4.8b	4.8ef	6.6ab	4.1abcd	3.5b	4.7ab	5.5bc	6.5ab	3.9b	3.3bc	4.8abc	6.7bcde	5.4abc
0.5	0.0	0.0	3.3de	3.4a	4.7b	5.4def	7.5a	3.9bcd	3.4b	5.2ab	4.5bc	6.4ab	4.2ab	2.9d	4.6abcd	6.9bcde	6.1a
1.0	0.0	0.0	3.8cd	3.5a	4.6b	6.2cd	6.0abcd	4.8a	3.4b	5.0ab	4.3c	5.8abc	4.3ab	3.3bc	4.9ab	5.9e	4.5cd
1.5	0.0	0.0	3.3de	3.3ab	4.2b	4.6f	5.7bcde	4.6ab	4.0a	5.3a	5.5b	5.6abc	4.6ab	3.5bc	4.7abc	6.2cde	5.4abc
2.0	0.0	0.0	3.1e	3.0bc	4.6b	4.8ef	4.7cde	4.0abcd	3.2b	4.5ab	5.9b	6.2ab	4.0b	3.5bc	5.2a	5.8e	5.9ab
0.0	0.58	0.0	3.1e	3.0bc	4.6b	4.6f	7.2a	3.7d	3.0b	4.4b	5.0bc	4.7c	5.0a	3.1cd	4.8abc	6.8bcde	5.2abc
0.0	1.16	0.0	3.4de	2.9c	3.5c	5.4def	4.8cde	4.3abcd	3.3b	4.9ab	5.8b	6.7a	4.4ab	3.5abc	3.9d	8.2abc	4.5cd
0.0	1.74	0.0	3.1e	2.7c	4.6b	5.0def	6.1abc	4.0abcd	3.2b	2.8d	5.3bc	6.5ab	4.4ab	3.8a	4.2abcd	9.9a	3.6d
0.0	2.32	0.0	3.5de	2.9bc	4.6b	6.0cde	4.7de	4.5abc	3.4b	2.9d	5.3bc	6.4ab	4.0b	3.4abc	4.5abcd	7.2bcde	6.2a
0.0	0.0	0.53	4.2bc	3.5a	5.6a	7.0c	5.3bcde	3.7cd	3.3b	2.4d	5.8b	5.8abc	4.5ab	3.5abc	4.6abcd	8.0abcd	4.6bcd
0.0	0.0	1.06	4.3ab	3.0bc	4.4b	7.4bc	5.8bcde	4.4abcd	3.2b	2.5d	5.5bc	5.8abc	4.5ab	3.2bcd	4.5bcd	8.5ab	4.6bcd
0.0	0.0	1.59	4.9a	3.4a	4.5b	8.9a	4.0e	3.8bcd	3.1b	3.6c	5.9b	3.2d	4.3ab	3.6ab	4.4bcd	7.0bcde	4.6bcd
0.0	0.0	2.12	4.5ab	3.4a	4.8b	8.7ab	5.7bcde	4.8a	3.0b	3.6c	7.7a	5.0bc	4.8a	3.4abc	4.1cd	6.0de	6.3a
SE±			0.057*	0.039*	0.061*	0.014*	0.026*	0.073*	0.0064*	0.011*	0.013*	0.014*	0.066*	0.0054*	0.061*	0.017*	0.122*

PH=Plant height; SD=Shoot diameter; NL=Number of leaves; NR=Number of roots; RL=Root length; SE± = standard error; *****=significant
Means followed by the same letters within a column do not differ significantly.

Acclimatization and Field Observation of *In Vitro* Rooted Plants

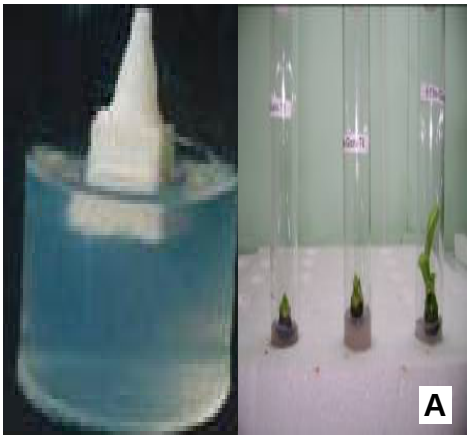
Well developed and healthy *in vitro* raised plantlets (about 6cm with 3-4 leaves) were transferred into small polybags filled with different soil media mix, and kept inside small chambers covered with transparent plastic in the lathouse for primary hardening for a week (Figure 1D). Plantlets were then transferred to big polybags filled with soil media for acclimatization (Figure 1E). All the plants were acclimatized (100% survival), and the highest vegetative plant growth in terms of plant height, pseudostem girth and number of photosynthetic leaf per plant was recorded on media containing sugarcane filter cake and sand at 3:1 ratio (v/v) as indicated in Table 4. Under Melkassa condition the acclimatization of plantlets took from 10 to 14 weeks depending on the type of banana cultivar. The hardened plants were transferred to the field, successfully established, and produced large bunches with high quality fruits (Figure 1F).

Table 4. Acclimatization of *in vitro* derived banana plants on different potting mix

Treatments	Plant height (cm)	Plant diam. (cm)	Leaf Numbers	Survival (%)
MS+S (3:1v/v)	5.05c	0.61c	5.20b	100
HS+S (3:1v/v)	5.53b	0.69b	5.95a	100
FC+S (3:1v/v)	7.84a	0.89a	5.95a	100
SE±	0.18*	0.020*	0.10*	

Key: MS=Melkassa soil; HS=Holleta soil; FC=Sugarcane filter cake; S=sand; v/v=volume to volume.
Means followed by the same letters within a column do not differ significantly.

In conclusion, as in many of the previous reports on banana micropropagation that used more than one type of media for initiation, multiplication and rooting (Cronauer and Krikorian, 1986; Jarret, 1986; Diniz et al., 1999; Nauyen and Kozai, 2001; Krishnamoorthy et al., 2001; Kagera et al., 2004), similar protocol is reported in the present study. Accordingly, MS medium with 3 mg/l BAP for Dwarf and Giant Cavendish while 2 mg/l for Poyo varieties can be used for initiation. For shoot multiplication, MS medium containing combinations of BAP and IAA at 3+0.4, 4+0.4 and 3+0.2 mg/l for Dwarf, Giant and Poyo respectively can be used. Furthermore, well rooted plantlets can be obtained when the shoots are cultured on MS medium with 2.12 mg/l NAA for Dwarf and Giant while 1.74 mg/l IBA for Poyo. The necessary vegetative growth can be attained for plantlets that are hardened and acclimatized on potting media with sugarcane filter cake and sand at 3:1 ratio (v/v). Therefore, the optimized protocol in the present attempt could be used for large scale massive banana plantlets production.



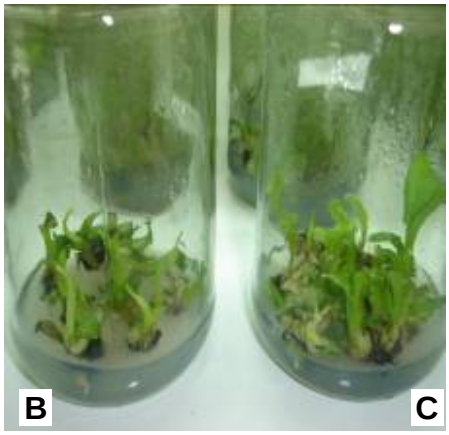


Figure 1. *In vitro* and *ex vitro* banana (A) Shoot tip initiation; (B) Shoot multiplication; (C) Shoot elongation and rooting; (D) Acclimatization inside chamber; (E) Acclimatized and hardened plants; and (F) *In vitro* derived plant at fruiting in the field

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