

In vitro propagation of the Orito banana cultivar (*Musa acuminata* AA)

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RESEARCH

ABSTRACT

Traditional banana propagation methods do not meet the demand of the crop or ensure the availability of disease-free plants. Moreover, the yield and productivity of the banana propagated by the traditional route is reduced due to the attack of diseases. *In vitro* micropropagation is the tool that allows obtaining plants with excellent characteristics: health, high vigor and fruit yield. Hence, in this research, a protocol was established for the *in vitro* propagation of the Orito banana cultivar (*M. acuminata* AA), comprising four phases (establishment, multiplication, rooting and acclimatization). Corms were used as planting material, the corresponding recesses were made before taking them to the laboratory. In the first phase (aseptic establishment of buds), contamination by microorganisms was efficiently controlled in T2 (0.1 % Tween 80 + 20 % chlorine + 0.2 % mercury bichloride for 5 min) obtaining 100 % contamination-free explants and 100 % survival. The healthy and aseptic explants were transferred to the multiplication phase, showing similar effects in the number of shoots (1.31) for all the concentrations studied, for the variable length of shoots T1 containing the lowest concentration (2 mg/L BAP + 0.43 mg/L IAA), a length of 8.57 cm was obtained. The resulting shoots were transferred to an *in vitro* cooling medium, and then to the greenhouse for rooting and *ex vitro* acclimatization using sand as substrate. In this last phase, 100 % survival, 7 roots per plant and an average height of 11.40 cm were obtained. The results were optimal and the plants generated by this technique were vigorous, healthy and aseptic.

Keywords: *In vitro* culture, propagation, greenhouse, banana, *Musa acuminata*

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RESUMEN

Propagación *in vitro* de banano variedad Orito (*Musa acuminata* AA). Los métodos de propagación tradicional de banano no satisfacen la demanda del cultivo ni garantizan la obtención de plantas libres de enfermedades o altos rendimientos al ser propagados por la vía tradicional, producto del ataque de enfermedades. La micropropagación *in vitro* es la herramienta que permite obtener plantas con excelentes características: sanidad, alto vigor y rendimiento de frutos. Por ello, en esta investigación se estableció un protocolo para la propagación *in vitro* de banano variedad Orito (*M. acuminata* AA), con cuatro fases (establecimiento, multiplicación, enraizamiento y aclimatación). Se partió de cormos, a los que se les realizaron los respectivos rebajes antes de llevarlos al laboratorio. En la primera fase de establecimiento aséptico de yemas se controló eficientemente la contaminación por microorganismos en el tratamiento T2 (Tween 80 0.1 % con cloro 20 % y HgCl₂ 0.2 % durante 5 min), obteniendo explantes completamente libres de contaminación y con un 100 % de supervivencia. Los explantes sanos y asépticos fueron transferidos a la fase de multiplicación, y mostraron efectos similares en el número de brotes (1.31) para todas las concentraciones estudiadas. La variable longitud de brote fue de 8.57 cm para el T1 (menor concentración de BAP 2 mg/L más AIA 0.43 mg/L). Los brotes obtenidos fueron sucesivamente transferidos a un medio de refrescamiento *in vitro* y al invernadero para su enraizamiento y aclimatación *ex vitro* en arena. En esta última fase se obtuvo un 100 % de supervivencia, 7 raíces por planta y una altura promedio de 11.40 cm, y las plantas obtenidas fueron vigorosas, sanas y asépticas.

Palabras clave: cultivo *in vitro*, propagación, invernadero, banano, *Musa acuminata*

Introduction

Orito banana or baby banana (*Musa acuminata* AA), is an edible product. In Ecuador, the cultivation of this species is very important for thousands of Ecuadorian families, due to the national and international demand, and it is exported to the European Union and US. The climatic conditions and the characteristics of the soil where orito banana is grown are adequate for the good development of this musaceae, where plantations are predominantly managed in an organic and traditional way [1].

Ecuador had 221 775 hectares of banana until 2012 [2], with an estimate of 8000 hectares growing *M.*

acuminata AA. Around 19 000 boxes of 16 pounds are exported weekly and 988 000 boxes annually with similar amount. About 10 tons of Orito banana are exported monthly to Europe; mainly to Rotterdam, in the Netherlands [3]. As a whole, 2011 was a good year for the export sector. The country sold 7176.7 tons of fruit to the world, generating revenues of US \$ 3.6 million, compared to US \$ 3.4 million in 2010 [4].

The cultivation of this musacea is carried out in the provinces of Guayas, Azuay, El Oro, Bolívar, Cotopaxi and Chimborazo. The largest extensions of this crop are located in the area of Bucay (Guayas).

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2. MAGAP. Serie histórica 2000-2012. 2015 [cited 2016 Aug 11]. Available from: <http://sinagap.agricultura.gob.ec/banano>

3. El banano orito despunta en los mercados. El Agro. 2014. 2014 Mar 31 [cited 2016 Aug 11]. Available from: <http://www.revistaelagro.com/2014/03/31/el-banano-orito-despunta-en-los-mercados>

Cultivation is predominantly organic and traditional [3]. However, traditional banana propagation methods do not meet the demand of the crop or ensure the availability of disease-free plants. In addition, the yield and productivity of banana propagated traditionally are reduced due to infestations, such as: black sigatoka (*Mycosphaerella fijiensis*), herenque (*Ralstonia solanacearum*), Panama disease (*Fusarium oxysporum*) which have risk the genetic resource for food and establishment of new crops [5].

In this scenario, micropropagation is a tool that allows obtaining large quantities of plants with good characteristics in terms of phenotype, genotype, establishment, high vigor and fruit yield. Moreover, it reduces the risk of dissemination of pests and diseases, as it occurs with the taking of plantlets (shoots) from already established plantations. *In vitro* micropropagation allows obtaining plants from a small fragment of tissue cultured under sterile conditions [6]. The *in vitro* culture of plant tissue is used as to overcome propagation difficulties and to avoid the extinction of valuable species [7]. This strategy has been applied by several researchers in different species obtaining good results for the massive propagation of musaceae [8-11].

In *Musa* spp., the beneficial effect of cytokinins on the formation of axillary shoots and on apical buds grown *in vitro* is known [12]. However, very little information is available on the *in vitro* propagation of Orito bananas by the use of whole corms. Given the importance of growing this species in Ecuador and improving plantations of small and medium producers, the objective of this research was to establish a protocol for the *in vitro* propagation of Orito banana cultivar (*M. acuminata* AA).

Materials and methods

Vegetal material

The vegetative material was obtained from Orito banana corms (*M. acuminata* AA) donated by the "Cruz" farm owned by Mr. Gelio Cruz, located in La Mana, Cotopaxi Province, Ecuador.

Culture media

The culture medium was MS [6] with 7 g/L bacto-agar as a gelling agent (Becton, Dickinson & Company) and 20 g/L sucrose. pH was adjusted to 5.7 with NaOH, then sterilized at 121 °C for 15 min. The inoculation and transfer of Orito meristems was carried out in the laminar flow cabinet.

In vitro propagation phases

Aseptic disinfection and establishment of buds

The whole corms of Orito underwent a manual cleaning process, eliminating the necrosed roots and parts with the help of razors, until reaching dimensions of 4 cm² approximately. After cleaning, they were placed in sterile distilled water with 0.1 % Tween 80 and 20 % chlorine for 20 min. Then, the explants were transferred to the laminar flow cabinet and a second recess was made, followed by disinfection treatment using Tween 80, 20 % chlorine and different concentrations of mercury bichloride (HgCl₂) (Table 1), making three rinses with sterile distilled water. Finally,

Table 1. Treatments and variables evaluated in the different *in vitro* propagation phases of Orito banana cultivar (*Musa acuminata* AA)

<i>In vitro</i> propagation phase	Label	Treatment		Variables
		Composition		
Establishment*	T1	0.1 % Tween 80 + 20 % chlorine + 0.1 % HgCl ₂		Contaminated explants (%) Explants without phenolization (%) Survival (%)
	T2	0.1 % Tween 80 + 20 % chlorine + 0.2 % HgCl ₂		
	T2	0.1 % Tween 80 + 20 % chlorine + 0.3 % HgCl ₂		
Multiplication	T1	2 mg/L BAP + 0.43 mg/L IAA		Shoots per explant Shoot length (cm)
	T2	3 mg/L BAP + 0.65 mg/L IAA		
	T3	4 mg/L BAP + 0.85 mg/L IAA		
Rooting and acclimatization		Sand (substrate)		Plant height (cm) Root length (cm) Roots Leaves Survival (%)

* Treatments were applied for 5 min. BAP: benzylaminopurine. IAA: indoleacetic acid.

the plant material was placed in 250-mL capacity flasks containing 20 mL of the semisolid MS medium, 10 mL/L ascorbic acid and 50 mg/L L-Cysteine were added to the medium to avoid the phenolization of the explants.

Multiplication culture medium

The type and different concentrations of growth regulators to improve the *in vitro* multiplication rate of Orito banana were evaluated in this phase. For performing this experiment, the aseptic shoots of the first phase were transferred to the MS multiplication medium with plant growth regulators (benzylaminopurine (BAP) and indoleacetic acid (IAA)) (Table 1). Then, the obtained shoots were then segmented and placed in liquid MS medium, containing 30 g/L of sucrose and 1 g/L of activated carbon for one month. The idea of using this medium was to generate a cooling and conditioning stage, so that the shoots increase in vigor before being taken to the rooting phase. Explants were exposed to continuous fluorescent white light (37 μmol/m² s) and temperature of 25 ± 2 °C.

Rooting and *ex vitro* acclimatization of vitroplants

For rooting and acclimatization, vitroplants from the cooling and *in vitro* conditioning stage were used as plant material. The vitroplants were placed in germinating trays with sand substrate (particles between 0.063 and 2 mm) without plant growth regulators, covered with a plastic tunnel to preserve the relative humidity and applying irrigation with microsprinklers at a one-minute frequency every hour, for four weeks.

Experimental design and statistical analysis

In order to analyze the test conditions in the establishment and multiplication stages, the completely randomized design (CRD) was used considering 3 treatments and 4 replicates each. The experimental unit consisted of four explants, with averages corresponding to 16 explants per treatment. All the evaluated variables were subjected to variance analysis to determine statistical significance; while for establishing the differences between the means of the treatments, the Duncan's Multiple Range Test at 95 % probability was used. In the rooting and acclimatization phase, descriptive statistical tests were applied,

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considering 15 vitroplants and the evaluation was performed at 28 days.

Results and discussion

Aseptic establishment of Orito buds

The aseptic establishment of buds was achieved by combination of T2 (0.1 % Tween 80 + 20 % chlorine + 0.2 % HgCl₂) for 5 min (Figure A), being effective for the control of pollutants like bacteria and fungi. These results are similar to those reported by other authors who, when using 20 % chlorine for 20 min plus 0.1 % HgCl₂ for 10 min obtained 100 % survival and contaminant-free shoots in the micropropagation of the Maqueño banana cultivar [13]. In the *in vitro* establishment of “Cambur Manzano” banana a high percentage of viable explants was obtained and low percentage of bacterial contamination, within the first week of cultivation [9].

Subsequently, 10 mg/L ascorbic acid and 50 mg/L L-Cysteine were added to the MS culture medium to avoid phenolization of the plant tissue, resulting in a low rate of explants with phenolization (6.25 %) in the T1 treatment, which implied obtaining a high percentage of survival (Table 2). In other works, the addition of 1 g/L of activated carbon and incubation in darkness for two weeks also minimized this phenomenon in apical meristem cultures of Maqueño and *Musa balbisiana* banana cultivars [13, 14]. Oxidations of explants were also noted during cropping of Harton apices [10], in explants in “Cambur manzano” banana exhibited a generalized oxidation in the tissue causing increased mortality [15, 16]. Oxidation in explants and contamination in the *in vitro* aseptic establishment stage are the main causes of loss of plant material that can be controlled with good disinfection and reducing oxidation with activated charcoal [8]. In this phase, suitable shoots were obtained for the *in vitro* multiplication of banana aseptic buds.

Multiplication phase

The addition of cytokinins and auxins for the regeneration of axillary buds in the multiplication phase promoted an increase in the rate of axillary tissue formation, the averages obtained were between 1.25 and 1.31 shoots (Figure B). These results indicate that the concentrations used in this research do not seem to be sufficient to promote significant sprouting of buds. These results were superior, for the multiplication phase of the “Cambur Manzano” banana (*Musa* AAB), obtaining 0.85 shoots per explant when using 5 mg/L of BAP, but by increasing the dose of BAP to (10 mg/L) obtained 2.3 shoots per explant [17]. In another research on “Cambur Manzano” banana cultivating apical buds in a nutrient medium with 5.0 mg/L of BAP, and cultivating buds extracted from whole corms with the same concentrations, 1.5 shoots were obtained per explant in both cases [9]; this low proliferation of shoots is associated with *M. balbisiana* cultivars that show low multiplication rate or no *in vitro* response [18]. The capacity of shoot proliferation in *M. balbisiana* also depends on the cultivar and clone [19].

These results are lower than those obtained by other authors who achieved a better response in the Maqueño banana cultivar propagation, by

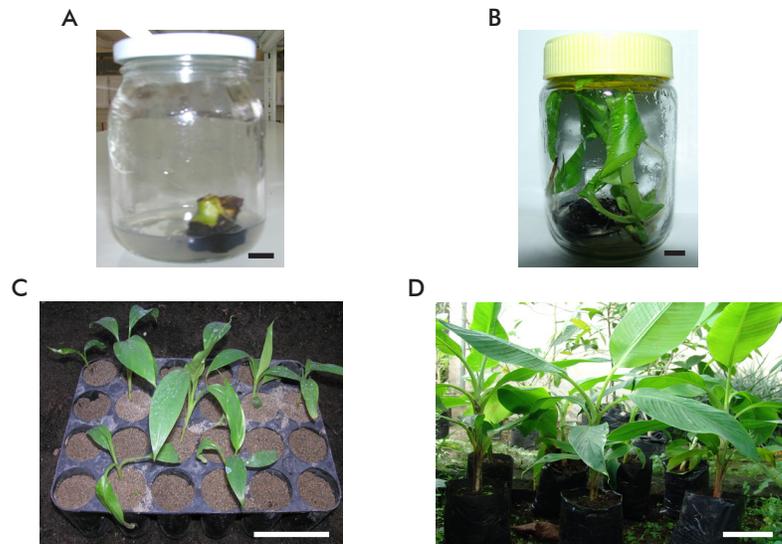


Figure. Average parameters of Orito banana cultivar (*Musa acuminata* AA) plants subjected to *in vitro* micropropagation procedures, during the establishment, multiplication and rooting and acclimatization phases. A) Banana explant in aseptic stage of establishment. B) Explants in the multiplication phase. C) Vitroplants in sand substrate. D) Acclimated banana plants. Estimation bars: A and B) 1 cm; C and D) 10 cm.

Table 2. Treatments and variables evaluated in the different *in vitro* propagation phases of Orito banana cultivar (*Musa acuminata* AA)

<i>In vitro</i> propagation phase	Treatment	Variables				
		Contaminated explants (%)	Explants without phenolization (%)	Survival (%)		
Establishment*	T1	18.75 a	93.75 a	75.00 b		
	T2	0 a	100.00 a	100.00 a		
	T2	6.25 a	100.00 a	93.74 a		
		Contaminated explants (%)	Explants without phenolization (%)			
Multiplication*	T1	1.25 a		8.57 a		
	T2	1.31 a		8.04 ab		
	T3	1.31 a		6.69 b		
		Plant height (cm)	Root length (cm)	Roots	Leaves	Survival (%)
Rooting and acclimatization	Sand substrate	11.40	6.28	7.00	4.83	100

* Average parameters with equal letters represent values without statistically significant differences ($p > 0.05$) according to the Duncan's test. Average correspond to 16 plants per treatment. In the establishment phase, T1 to T3 treatments corresponded to 0.1 % Tween 80 plus 20 % chlorine and 0.1, 0.2 or 0.3 % HgCl₂, respectively, all the treatments applied for 5 min. Likewise, treatments T1 to T3 in the multiplication phase were 2 mg/L BAP plus 0.43 mg/L IAA (T1), 3 mg/L BAP plus 0.65 mg/L IAA (T2) and 4 mg/L BAP plus 0.85 mg/L IAA (T3). BAP: benzylaminopurine. IAA: indoleacetic acid.

supplementing with 5 mg/L BAP + 1.2 mg/L IAA, the average multiplication rate was 2.5 shoots [13]. Regarding shoot length, the best treatment was T1 for an average of 8.57 cm. This coincided with another report on the obtainment of larger microstems by culture medium supplementation with a lower concentration of cytokinins, since this is achieved by decreasing cell division and promoting tissue elongation by the action of auxins [20]. It is important to mention that as the concentration of cytokinins (BAP) in the culture medium increases, the length of the shoot decreases (Table 2; multiplication phase). Other authors indicate that in *Musa* spp., the beneficial effect of cytokinins on the formation of axillary shoots on apical buds grown *in vitro* is known [11, 12]. At this stage, the presence of contaminating microorganisms was not detected, the same result as obtained in *Musa*

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acuminata (Simmunds) and “Cambur Manzano” banana [8, 9].

Acclimatization and rooting phase

Once the *in vitro* propagation of the seedlings has been completed, it is essential to adapt them to the uncontrolled *ex vitro* environmental conditions, both at cultivation houses and under field conditions [21]. Plants must adapt themselves both morphologically and physiologically after their transfer from *in vitro* culture to *ex vitro* conditions, that is to say, when changing their heterotrophic or mixotrophic metabolism to the autotroph one [22].

Transfer from the *in vitro* to *ex vitro* phase is the determining factor where high risks of seedling mortality are verified. In this work, the two phases of acclimatization and rooting of shoots were developed under greenhouse conditions, using germinating trays and sand as substrate without addition of plant growth regulators (Figure C). This phase was previously implemented by another researcher who used rooting beds with sand substrate under a sprinkler system and 50 % shade [23]. Banana seedlings were not subjected to addition of rooting regulators (exogenous auxin), and 28 days after the shoots were established, 100 % survived, with vitropplants of average height 11.40 cm, 6.28 cm of root length, 7 roots, and an average of 4.83 leaves each (Table 2; rooting and acclimatization phase). The substrate employed allowed a uniform distribution of O₂ that favored the development of the seedling root system. Availability of O₂ is essential for the development of the root system, where its demand is greater at 21 °C due to the metabolism required by the plant [24].

The results obtained in this phase of the research exceeded those obtained by other authors who worked on the propagation of the Maqueño banana cultivar, the results obtained were: 6.10 cm of root length, 10.7 cm height and 80 % survival [13]. At two months, the plants were completely acclimated, ready to be established in the field (Figure D).

The *in vitro* propagation protocol of Orito banana apical buds from corms obtained from a plantation, represents a contribution to the micropropagation of this species and generates the knowledge for the development of future studies tending to improve the multiplication rate. With the protocol used during the *in vitro* establishment phase, contamination-free and viable explants were obtained to continue the propagation process. The number of shoots obtained per explant in the multiplication phase was 1.31. The results obtained when evaluating the two phases together, acclimatization and rooting of the shoots under greenhouse conditions, using germination trays and sand as substrate without addition of growth regulators, were excellent. This allowed completing successfully the entire *in vitro* propagation process, as well as saving time and resources.

Conclusions

By means of the *in vitro* micropropagation protocol used in the present research, it was possible to establish aseptic buds of Orito banana (*Musa acuminata* AA). In the multiplication phase, the concentrations of BAP plus IAA showed similar effects in all evaluated treatments. However, they favored the sprouting of buds and bud elongation. The use of germinating trays with sand substrate in the rooting and *ex vitro* acclimation phases allowed obtain vigorous, healthy, aseptic plants with 100 % survival. The Orito banana plants obtained by *in vitro* propagation developed normally and after two months were ready for field establishment.

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