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Micro-propagation Factors Essential for Mass Production of Synthetic Seeds in Banana

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Abstract

This work described some essential factors necessary for micro-propagation of banana for mass production of synthetic seeds for germplasm conservation, and how peroxides activity of conserved tissue was influenced. Shoot tips of field grown plants were used to obtain shoot clusters on shoot proliferation medium (MS medium supplemented with 5 mg/l BAP). Using longitudinally-split shoot tip technique, 18720, 8640, 7488, 2016 plantlets were obtained from one shoot tip of Maghraby, Grand Naine, Balady, and Williams, respectively, in six subculture, one month each, on solid medium. Shoot tips excised from *in vitro* grown plantlets were encapsulated in calcium-alginate beads and stored at 4℃ for one month on half-strength MS basal medium without growth regulators or sugars. After one month all the viable-conserved synseeds formed shoots when they were transferred to MS basal medium, some of them showed synchronous formation of shoot and root systems in one week. Plants retrieved from encapsulated shoot tips were hardened off and transferred to soil.

Key words: Artificial seeds, germplasm conservation, micro-propagation.

Introduction

Banana is one of the most important crops in the world. Pseudostem of banana plant formed from leaf sheathes which encircle each other on massive underground corms with highly compressed internodes. The growing point is located at the center of the corm. During flowering the internodes lengthen and the true stem is pushed up through the center of the pseudostem to form a long peducle (Barker and Steward 1962). Since banana cultivars never set seeds, they are propagated vegetatively by suckers and conserved in field gene banks. The latter purpose requires much area of land, consequently, establishment of banana tissue collection consisting of shoot cultures are an attractive alternatives for field long-term conservation. In breeding, plantlets obtained from banana tissue culture are better than those obtained from conventional methods because they show high survival rate, uniform and vigorous growth, having a shorter harvesting period and reduce the cost of disease and pest control.

Artificial seeds (synthetic seeds = synseeds) have been defined as an artificially encapsulated somatic embryos or non-embryogenic *in vitro*-derived vegetative propagules which can be used for sowing under *ex vitro* or *in vitro* condition, that are able to grow into plantlet after sowing (Bapat 1993; Standardi and Piccioni 1998; Adriani et al. 2000). The use of artificial seeds makes the tissue culture techniques more advantageous due to rapid multiplication, maintained genetic uniformity of plants, direct delivery of propagules to the field, thus eliminating transplants and omitting the acclimatization steps, reduction of storage space, reduction in costs of vegetative propagated plants and reduction in the breeding cycle (Maruyama et al. 1996). In most cases, artificial seed production depended on encapsulation of somatic embryo in a protective coat (Ganapathi et al. 1992; Nieves et al. 1998; Ganapathi et al. 2001). Only few reports described successful encapsulation of non-embryogenic *in vitro*-derived vegetative propagules such as maxillary buds or shoot tips (Sharma et al. 1994; Piccioni and Standardi 1995; Maruyama et al.1997;

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Patnaik and Chand 2000). In addition, while *in vitro* germplasm conservation has been published for many species (Withers 1985; Ng and Ng 1991; Dodds and Roberts 1995; Houwe et al. 1995, Brischia et al. 2002), conservation via encapsulation techniques have been reported for only a few species (Ganapthi et al. 1992; Standardi and Piccioni 1998; Pattnaik and Chand 2000).

Although micro-propagation of banana has been extensively used to many cultivars, many obstacles remain to be overcome before an effective banana regeneration protocol suitable for mass-production of synseeds is developed. Therefore, the objective of the investigation reported here was to study some factors affecting on banana *in vitro* multiplication to obtain high number of shoot tips in short time, which is suitable for mass-production of synthetic seeds. In addition, the effect of germplasm conservation of banana via shoot tip encapsulation on peroxidase activity was investigated.

Materials and methods

Plant materials and establishment of shoot cultures

In this work, four banana cultivars were used (Grand Naine, Williams, Maghraby and Balady). Cultures were established by isolating shoot apices from field grown plants (1-1.5 m in tall). After separating the suckers from the mother plants, the outer leaf sheathes and corm tissue were trimmed to get cylinder-shape cuttings (6 cm in length, 2 cm in diameter), with the shoot-tip at the center. These cuttings were disinfected by dipping in 5 % Clorox solution for 5 min followed by 5 min dip in 0.1% (w/v) Hg Cl_2, then they were rinsed three times with sterile distilled water. After disinfections, the excessive tissues were removed to get 5-7 mm shoot tip explants. These explants were cultured on shoot multiplication medium.

In vitro shoot cultures were obtained on basal MS (Murashige and Skoog 1962) medium supplemented with 5 mg l^{-1} BAP and 3 % sucrose. The medium was solidified with 8 g agar/l. Vitamins (mgl^{-1})) were: myo-inositol (100), vitamin b hydrochloride (4), nicotinic acid (4), pyridoxal hydrochloride (0.7), biotin (0.04) and colic acid (0.5). The pH of the medium was adjusted to 5.8 prior to autoclaving for 20 min at 120℃. The shoot cultures were maintained in 16-h photoperiod (irradiance of 60 μmol m^{-2} s^{-1}) using white fluorescent lamp at temperature of 25 ± 1℃.

Shoot proliferation as influenced by cutting or splitting the shoot tips longitudinally

Shoots tips obtained from field grown plants were cut, one time, vertically in such a way as to keep the base of the explants intact. On the other side, the shoot tips obtained from the *in vitro* grown shoots were splited longitudinally and cultured the resulting halves as individual explants in such a way to keep the cut side in contact with the medium surface. Also, the number of shoots/ explants and the weight of individual shoot were determined. The experiment was repeated three times each one was done using 18 explants cultured in three glass jars.

Shoot proliferation as influenced by media texture

Shoot tips were prepared from *in vitro* grown shoots as previously mentioned and cultured on liquid, semi-solid (containing 4 g /l agar) and solid (containing 8 g /l agar) shoot proliferation media. After 30 days culture, the number of shoots/explant and the weight of individual shoot were determined. This experiment was repeated three times, each one was done using 18 explants cultured in three glass jars.

Shoot proliferation as influenced by the source of explants

To study the effect of the source of explants on shoot proliferation, shoot-tips were cut from newly formed buds, short shoots (1.5 cm high) or shoots (5 cm high) and cultured on shoot multiplication medium, the number of shoots/explant and the weight of individual shoot were determined. This experiment was repeated three times, each one was done using 18 explants cultured in three glass jars.

Encapsulation of shoot tips

Sodium alginate (gel matrix) was added at concentrations of 3, 3.5 and 4 % to liquid shoot proliferation medium with or without 2 g/l activated charcoal. For complexation 75 mM $CaCl_2$ solution (complexing agent) was prepared using shoot proliferation medium. Both the gel matrix and complexing agent were autoclaved at 120℃ for 20 min and stored at room temperature. Encapsulation was accomplished by dipping the shoot tips with forceps in gel matrix, followed by dropping the shoot tips into the complexion agent for 30 min. Alginate beads containing shoot tips were collected and rinsed twice for 10 min each in water to wash away

calcium chloride residues. The obtained beads were 5-6 mm in diameter.

Synthetic seeds storage

A set of encapsulated shoot tips were conserved in empty-closed glass jars or in glass jars containing 25 ml half-strength basal medium (MS) without growth regulators and sugar, and stored in a laboratory refrigerator at 4℃. A group of un-encapsulated shoot tips were stored under the above condition to serve as a control. Viability of shoot tips was tested weakly for four weeks. The shoot tips were considered alive if the shoot tips were still green, with no necrosis. For each treatment 30 shoot tips were used.

Peroxidase activity

Encapsulated shoot tips cultured on basal medium (MS) at 25℃ with or without 2% activated charcoal were collected after 15 days to study the effect of charcoal on peroxidase activity of the cultured tissue. In addition, the effect of 2% charcoal in gel matrix on peroxidase activity of the encapsulated tissue was investigated (See table 5).

Estimation of relative peroxidase activity was measured (O.D./g fresh weight/ hr) and calculated according to Wakamatsu and Takahama (1993) and Kar and Mishra (1976). For peroxidase analysis 0.5 g of shoot tips extracted from capsules was ground at 4℃ in a mortar in 0.5 ml extraction buffer consisting of 0.1 M Tris - HCl pH 7.0 and containing 0.002 M cysteine. The homogenate was centrifuged at 15000 g at 4℃ for 15 min. The supernatants were collected for immediate peroxidase activity determination. The reaction mixture consisted of 5 mM guaicol, 40 mM potassium phosphate buffer, pH 7.2, 0.1 mM EDTA, 0.3 mM H_2O_2 (Wakamatsu and Takahama 1993).

Plant regeneration from encapsulated shoot tips

Stored encapsulated shoot tips were transferred to basal MS medium for germination under tissue culture room condition. Plantlets showed extensive root systems were transferred to soil. Plantlets obtained from adventitious buds or encapsulated shoot tips were transferred to plastic pots (10 cm in diameter) containing mixture of sand : beat (1:1) moistened with tap water. Immediately, the pots were covered with polyethylene bags to maintain a high humidity for one week. Then, the polyethylene bags were pored and the diameters of the pores were increased each two days for another week. After 60 days, the plantlets were transferred to garden.

Results and Discussion

Shoot tips were collected from sword suckers, 100-150 cm high. After leaf sheathes and corm tissue were removed, cylinder-shape cutting including the shoot-tip at the center was obtained (Fig. 1 A). This plant material was contaminated with micro-organisms, the sterilization procedure outlined in this work resulted in contamination rates of less than 5%. The disinfected shoot tips (0.5-0.6 cm) were placed directly onto medium that encouraged shoot multiplication (Fig. 1 B), it was MS medium supplemented with 5 mg/l BAP. As it has been previously reported, the most important factor in banana multiplication *in vitro* was clearly the cytokinin concentration (Lee 2003; Gübbük and Pekmezcl 2004). All the used cultivars in this work proliferated profusely on medium containing 5 mg/l BAP (Table 1).

In this work, preliminary experiments indicated that 0.5-0.7 cm shoot tip cuttings were the best size of explants, where the survival rate was 100% and the number of

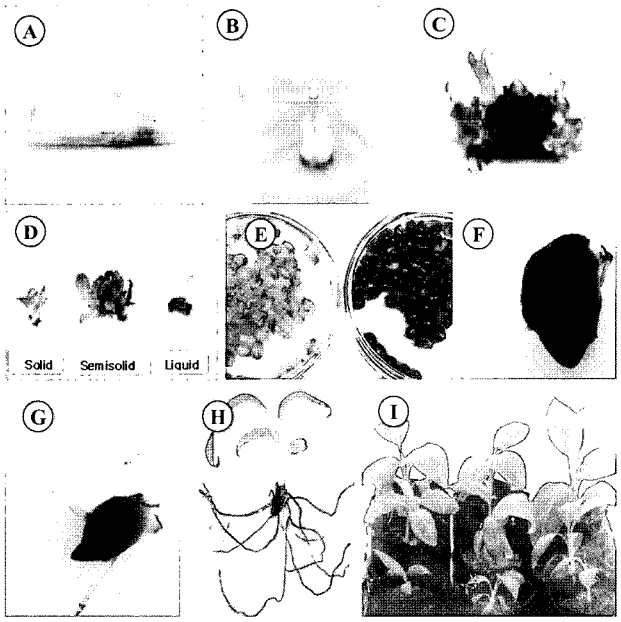

Figure 1. Photographs show banana propagation and synthetic seed production: (A) cylinder-shape cuttings after removal of successive leaf bases and ready for sterilization, (B) shoot-tip explant was longitudinally split and subcultured on shoot proliferation medium, (C) the lateral buds formation on the excised shoot-tip, (D) the shoots multiplication rate of a banana variety (Williams) as influenced by medium texture, (E) banana synthetic seeds (right: endosperm containing activated charcoal), (F) early germination of synthetic seed (in one week), (G) germinated seed with shoot and roots (in two weeks), (H) banana shoot with extensive root system, (I) hardened plants ready for transfer to the field.

obtained shoots per explant were relatively high (Table 1). Very small shoot tip explants may consist of nothing more than the apical meristem dome which increases the likelihood of mortality rate and drastically reduces the growth rate. On the other hand, the large explants (0.7-1.0 cm) showed more blackening and contamination resulting in low survival rates. The present studies are in agreement with the results reported previously (Swamy et al 1983; Krikorian and Cronauer 1984; Vuylsteke 1989). Shoot-tips cultured on multiplication medium turned green in 7-10 days and swelled at the base in 2-3 weeks. Adventitious buds were detected in 3-4 weeks. Sequential removal of the remaining leaf initials was necessary to obtain more buds. With time, the cultured shoot tips formed clusters of small shoots (Fig. 1 C), which were subcultured to obtain shoot tips for encapsulation experiments.

The multiplication rate of adventitious shoots depended on cultivar and the number of subcultures (Table 1). In general, explants obtained from field grown plants (GO) formed more adventitious shoots (Fig. 1 C) than those obtained from *in vitro* grown shoots (Fig 1. D), and it depended on the cultivar. Number of adventitious shoots in

Table 1. Effect of repeating subculture at interval of one month and ascorbic acid (200 mg/l) on the multiplication rate of different banana varieties (Williams, Grand Naine, Maghraby and Balady). Go: explants were obtained from field grown plants and cultured for two months. Values are mean ± SD.

Variety	No. of subcultures	Treatment			
		Control		ASA	
		No. of shoots / explant	F. wt.(g) / shoot	No. of shoots / explant	F. wt.(g) / shoot
Williams	Go	7 ± 1.16			
	G1	3 ± 0.71	0.12 ± 0.00	4 ± 1.18	0.10 ± 0.03
	G2	4 ± 1.15	0.13 ± 0.06	6 ± 1.15	0.16 ± 0.07
	G3	3 ± 0.14	0.13 ± 0.03	4 ± 0.93	0.08 ± 0.01
	G4	2 ± 0.96	0.07 ± 0.02	2 ± 0.00	0.04 ± 0.004
	G5	2 ± 0.06	0.12 ± 0.009	2 ± 0.00	0.04 ± 0.02
	G6	3 ± 0.12	0.23 ± 0.04	2 ± 0.00	0.04 ± 0.02
Grand naine	Go	4 ± 0.76			
	G1	5 ± 0.94	0.28 ± 0.04	6 ± 1.53	0.16 ± 0.09
	G2	4 ± 0.49	0.20 ± 0.01	4 ± 0.29	0.11 ± 0.03
	G3	4 ± 0.47	0.10 ± 0.01	2 ± 0.82	0.04 ± 0.02
	G4	3 ± 0.51	0.10 ± 0.02	2 ± 0.14	0.03 ± 0.006
	G5	3 ± 0.58	0.14 ± 0.02	2 ± 0.58	0.08 ± 0.009
	G6	3 ± 0.71	0.12 ± 0.005	1 ± 0.00	0.08 ± 0.01
Maghraby	Go	13 ± 1.76			
	G1	5 ± 0.24	0.13 ± 0.09	4 ± 0.23	0.08 ± 0.02
	G2	6 ± 0.84	0.17 ± 0.004	4 ± 0.51	0.15 ± 0.01
	G3	4 ± 0.94	0.15 ± 0.009	4 ± 0.82	0.10 ± 0.02
	G4	2 ± 0.19	0.09 ± 0.01	3 ± 0.12	0.08 ± 0.01
	G5	2 ± 0.00	0.07 ± 0.006	2 ± 0.71	0.05 ± 0.01
	G6	3 ± 0.76	0.12 ± 0.03	2 ± 0.29	0.10 ± 0.03
Balady	Go	13 ± 1.06			
	G1	3 ± 1.06	0.10 ± 0.01	4 ± 1.06	0.04 ± 0.01
	G2	4 ± 0.71	0.20 ± 0.08	5 ± 0.50	0.14 ± 0.04
	G3	4 ± 0.58	0.13 ± 0.04	4 ± 0.58	0.10 ± 0.01
	G4	2 ± 0.18	0.05 ± 0.008	2 ± 0.00	0.02 ± 0.003
	G5	2 ± 0.58	0.10 ± 0.01	1 ± 0.00	0.04 ± 0.009
	G6	3 ± 0.23	0.17 ± 0.02	2 ± 0.35	0.07 ± 0.01

GO of Maghraby and Balady varieties (13 shoots) were higher than those of Williams (7 shoots) and Grand naine (4 shoots) varieties. During the first (G_1), second (G_2) and third (G_3) subcultures, the number of shoots remained more or less unaffected. However, during the following subcultures (G_4-G_6), the number of shoots exhibited, in most cases, somewhat lower values compared with those of the first three subcultures. In this concern, Vuylsteke (1989) reported that proliferation rates increased with the number of subculture, should be limited to six or seven in order to reduce the incidence of off-types arising through somaclonal variation (Krikorian and Cronauer 1984, Roels et al 2005).

Shoot-tip explants were inoculated onto multiplication medium as fragmented shoot apex (Fig 1 B). Longitudinal cut of shoot-tips explants, obtained from *in vitro* grown plants, duplicated the number of shoots/ shoot tip (Table 2), it may be due to reduce apical dominance (Krikorian and Cronauer 1984; Vuylsteke 1989). Using this technique, 18720 plantlets in Maghraby, 8640 plantlets in Grand naine, 7488 plantlets in Balady, and plantlets 2016 in Williams were obtained, in six subcultures on solid medium, in seven months.

The effect of shoot-tip explant source on the adventitious shoot formation was also studied (Table 3). The number of adventitious shoots was higher when the explants were obtained from field grown plants than those obtained from *in vitro* grown shoots. In addition, the number of adventitious shoots obtained from 5 cm high shoots was better than 0.5 cm high shoots. The effect of explants source was studied by many authors (Durzan 1984; Hassanein and Mazen 2001; Hassanein 2004).

Successful *in vitro* culture depended largely on chemical composition and physical form of the medium. While MS medium is very suitable for banana shoot-tip culture, it depended greatly on medium texture, solid, semi-solid, or liquid. The data in our work (Table 4) indicated that semi-solid medium was better than liquid or solid medium, where the highest number of buds were obtained in all the used cultivars. In few cases, the use of liquid medium was pre-

Table 2. Effect of splitting the explant vertically on the multiplication rate of banana plants (Maghraby) in comparison to the intact explant. Values are mean ± SD.

Type of explant	No. of shoots / explant	F. wt.(g) / shoot
Intact shoot tip	3 ± 0.81	0.71 ± 0.04
Longitudinal section from the shoot tip	6 ± 1.52	0.19 ± 0.06

Table 3. Effect of the explant source on the multiplication rate of banana plants (Maghraby) Values are mean ± SD.

Source of explant	No. of shoots / explant	F. wt. (g)/ shoot
1.5 - 5 high shoot	3 ± 0.81	0.71 ± 0.04
0.5 cm small bud	1 ± 0.45	0.13 ± 0.009

Table 4. Effect of different solidification states of MS medium (supplemented with 5 mg/l BAP) on the multiplication rate of different banana varieties (Williams, Grand naine, Maghraby and Balady). Values are mean ± SD.

Vareity	Solidification state	No. of shoots / explant	F. wt.(g) / shoot
Williams	Solid	4 ± 0.00	0.14 ± 0.02
	Semisolid	5 ± 0.88	0.41 ± 0.06
	Liquid	1 ± 0.35	0.31 ± 0.04
Grand nains	Solid	3 ± 0.28	0.13 ± 0.01
	Semisolid	4 ± 0.26	0.16 ± 0.03
	Liquid	1 ± 0.00	0.08 ± 0.004
Maghraby	Solid	4 ± 0.76	0.12 ± 0.03
	Semisolid	3 ± 0.37	0.27 ± 0.06
	Liquid	1 ± 0.00	0.16 ± 0.01
Balady	Solid	3 ± 0.47	0.13 ± 0.04
	Semisolid	4 ± 0.23	0.18 ± 0.07
	Liquid	1 ± 0.00	0.14 ± 0.04

Table 5. the effect of 2% charcoal in MS basal medium or in gel matrix on peroxidase activity of the encapsulated tissue.

Temperature (℃)	Shoot tips cultured on MS medium without growth regulators	
25	MS without charcoal (Control)	MS with 2 g/ l charcoal
	100 ± 00	104 ± 10
	Encapsulated shoot tips conserved in empty glass jars	
	Capsule matrix without charcoal	Capsule matrix with 2 g/l charcoal
25	86 ± 5	107 ± 1
4	69 ± 3	82 ± 4
	Encapsulated shoot tips conserved on conservation medium	
	Capsule matrix without charcoal	Capsule matrix with 2 g/l / charcoal
4	64 ± 3	65 ± 2

ferable (Krikorian and Cronauer 1984; Novak et al. 1986).

Encapsulation of 0.5 cm shoot tips using 3.5 % sodium alginate upon complexation with 75 mM $CaCl_2$ produced firm and uniform capsules within ion exchange duration of 30 min and it did not depend on the presence or absence of activated charcoal (Fig.1 E). After one month, the percentage of viable synseeds was higher when they conserved on medium (96%) than those conserved in empty glass gars (35%). On the other side, a control shoot tip (without embedding in calcium alginate capsules) was completely damaged after four days in lab. refrigerator. Shoot cultures are very suitable material for local and international movement as well as *in vitro* conservation of *Musa* germplasm. In addition storage of alginate-encapsulated shoot tips at low temperature (4℃) was found to be an acceptable method for conservation of germplasm cultured *in vitro* (Maruyama et al. 1997), where it abolished growth due to a reduction of the respiration process in encapsulated tissue (Brodelius et al. 1982).

After one month all the viable-conserved synseeds formed shoots when they were transferred to MS basal medium, some of them showed synchronous formation of shoot and root systems in one week (Fig 1 F and G). With time, all shoots formed extensive root system (Fig. 1 H). Plants retrieved from encapsulated shoot tips were hardened off for two weeks under room conditions using plastic bags and transferred to garden (Fig. 1 I).

Plants obtained from encapsulated shoot tips had normal phenotypes and grow well in the garden (Fig 1 I). It gives good indication about the suitability of encapsulated shoot tips for the purpose of germplasm conservation more than *in vitro* germplasm conservation. Reduced temperature significantly increased the subculturing interval (Van den Houwe 1995). While it permanently threatened by the occurrence of somaclonal variation (Côte et al. 1996), it may be lower under germplasm conservation conditions than normal tissue culture conditions.

Enhancement of peroxidase activity (Table 5) was detected in encapsulated shoot tips in comparison to that of control. It seems to reflect, with reasonable accuracy, the biological modification of the encapsulated issue inside the capsule, where, germplasm conservation via encapsulation technique should be stressed to abolish greatly the growth and organogenesis. This situation gave additional indication about existence of close relation between peroxidase activity and conditions stimulating or abolishing growth and organogenesis (Senaratna 1992; Booij et al., 1993; Redenbaugh 1993; Hassanein et al. 1999; Hassanein 2004). The peroxidase activity was influenced by the temperature of conservation or the presence or absence of activated charcoal in capsule matrix. In general, decreasing the temperature from 25 to 4℃ was accompanied with a decrease in peroxidase activity. At any temperature degree, incorporation of charcoal in capsule matrix increased peroxidase activity.

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