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Factors Affecting Synseeds Formation and Germination of Banana Cultivar Grande Naine

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Abstract: Synthetic seeds technique has unraveled new vistas in *in vitro* plant biotechnology, such as large scale clonal propagation, delivery of clonal plantlets, germplasm conservation, breeding of plants in which propagation through normal seeds is not possible, genetic uniformity, easy storage and transportation etc. This study aimed to optimize a method of banana synthetic seeds formation and germination. Analysis data conducted that concentrations of sodium alginate and calcium chloride, exposure times to the later, BA concentration in alginate matrix and composition of alginate matrix were affected formation and germination of banana synthetic seeds. Results cleared that synthetic seeds formation and texture depended on both Na-alginate and $CaCl_2.2H_2O$ concentrations. The more rounded and good texture was obtained from 4% sodium alginate when it dropped in 100 mM $CaCl_2.2H_2O$**.** Exposure times to $CaCl_2.2H_2O$ affected synthetic seeds germination, exposure for ten minutes to $CaCl_2.2H_2O$ resulted in 100% germination after 16 weeks. Banana synthetic seeds germination increased with increasing of benzyl adenine (BA) in sodium alginate matrix, from 80% synthetic seeds germination at control (free of BA) to 100% synthetic seeds germination at 5.0 mg/l BA. Adding 2.5 mg/l BA, 1.0 g/l activated charcoal (AC) and MS medium to sodium alginate matrix enhanced banana synthetic seeds germination compared with distilled water, MS or MS+2.5mg/l BA (97.1, 71.4, 85.7 and 92.9% of synthetic seeds germination, respectively). Also, growth proliferation and growth parameters after germination were affected by synthetic seeds composition. Results of genetic analysis of banana synthetic seeds using RAPD and ISSR genetic markers, which resulted in producing 51 total amplified fragments from ten RAPD primers and 28 total amplified fragments from ten ISSR primers, revealed that synthetic seeds is a save method to produce true to type plantlets.

Key words: Activated charcoal (AC) · Banana · Benzyl adenine (BA) · Calcium chloride · Genetic stability · Sodium alginate · Synthetic seed germination

INTRODUCTION

The first definition of synthetic seeds (or artificial seeds or synseeds) was as "an *encapsulated* single somatic embryo" [1]. The increasing number of studies were focused on the use of non-embryogenic propagules for the manufacture of synthetic seeds allowed to extend their definition as "artificially *encapsulated* somatic embryos, shoot buds. In addition to the other *in vitro* derived meristematic tissues like microtubers, rhizomes and corms can also been used as functionally mimic seeds for sowing and possessing the ability to evolve into plantlets (*conversion)* under *in vitro* or *ex vitro* conditions, which can be retained even also after storage" [2, 3]. Production of synthetic seeds has unraveled new vistas in *in vitro* plant biotechnology, such as large scale clonal propagation, delivery of clonal plantlets, germplasm conservation, breeding of plants in which propagation through normal seeds is not possible, genetic uniformity, easy storage and transportation etc [4] because artificial seeds technology offers several potential advantages: (1) ease of handling, (2) low production cost, (3) ease of exchange of plant materials between different laboratories in different counties, (4) genetic uniformity of propagated plants (5) direct delivery to the soil, (6) shorten the breeding cycle and (7) reduction of the storage space

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[5-7]. The encapsulated shoot tips can be handled like a seed and could be useful in minimizing the cost of production as 1 ml of medium is sufficient for encapsulation of a single shoot tip compared to 15-20 ml for conversion of shoot tips into plantlets [8, 9]. By directly sowing the encapsulated shoot tips in soil, the two stage process such as rooting and hardening can be eliminated. As compared to suckers, encapsulated shoot tips present as inexpensive, easier and safer material for germplasm exchange, maintenance and transportation [10].

The shape and texture of the beads depends on the concentration of the sodium alginate, calcium chloride solutions and duration of complexion. The main advantages of Na-alginate are: the excellent water solubility and moderate viscosity of Na-alginate at room temperature, its easy availability at low cost, the long-term storability of the Na-alginate solution, the easy use of calcium salts for quick gellation and bead hardening at room temperature, the possibility to prepare synseeds of different hardness by changing the concentration of Na-alginate and/or the duration of the ion-exchange reaction, the absence of any kind of toxicity of the Ca-alginate matrix for explants, the possibility to mix the alginate with a nutritive medium to obtain an artificial endosperm [11]. Also, Winkelmann *et al*. [12] conducted that synthetic seed coat should be able to deliver the nutrients, the growth regulators and the other components of the artificial endosperm which are necessary for the germination of the synseeds, while at the same time to enable handling during storage, transportation and planting. They added that the hardening of beads is generally performed in 50-100 mM of $CaCl_2.2H_2O$, with exposure times ranging from 20 min for *Cyclamen persicum* to 60 min for *Pelongonium horturum* [13]. The highest germination rate was obtained when a 30-min exposure to 50 mM $CaCl_2.2H_2O$ was applied for bead polymerization, in comparison with a 10-min exposure to 60 mM or 80 mM [14]. In Orchids, the most important cut flowers which are commercially propagated *in vitro*, the best conversion to plantlets (100%) of encapsulated of *Dendrobium* 'Sonia' was observed when 3% Na-alginate drops were hardened for 30 min in 75 mM $CaCl_2.2H_2O$ [15]. Shoot tips stored for 12 weeks and encapsulated in 3% sodium alginate prepared in distilled water without MS medium [16]. In other studies use of meristematic shoot tips or axillary buds were used for the production of synthetic seeds as reported for banana [17, 18].

BAP in high concentration with lower concentration of NAA increased germination of beaded embryos over control. High percent of germination (55-87.5%) was observed when MS was supplemented with BA and lower concentration of NAA, whereas, addition of Kn in MS reduced the germination percentage [19]. The germination percentage of encapsulated microshoots was affected by percentage of sodium alginate (2%, 3% and 4%), encapsulation matrix and duration of exposure to $CaCl_2.2H_2O$ solution [20, 21]. Synthetic seeds techniques may improve the quality of banana (*Musa sp*) plantlets and decreasing the production costs. This technique was successfully employed in the cvs. ''Rasthali' by Ganapathi *et al*. [6], Basrai' by Ganapathi *et al*. [17] and 'Nanicão' by Matsumoto *et al*. [22] and resulting in high conversion rates (100%, 75% and 66%, respectively). In summary, the protocol for the production of banana synthetic seeds of microshoots includes the use of alginate capsules containing sucrose-free MS salts, activated charcoal (1.5 g/l) and benomyl (0.5g/l) and KNO_3 solution (100mM) treatment for 20min for weakening of the capsules [23].The molecular studies to determine genetic stability of synthetic seeds derived plantlets were started from the last decade, but no modifications were revealed at the biochemical and/or molecular levels. The potential advantage of synthetic seeds for genetically identical to natural plants was supported by Nyende *et al*. [24]. The genetic stability of plantlets derived from encapsulated *Ananas comosus* microshoots was proved by random amplified polymorphic DNA (RAPD) and ISSR techniques [25].

The present study focused on the presentation of a method used to produce the artificial seed of banana cultivar Grande Naine from *in vitro* microcorms (microshoots) and investigated factors affecting short term conservation and synseeds germination after conservation. Also, genetic stability examination of banana synthetic seeds, which is essential perquisite for success this technique, was determined.

MATERIALS AND METHODS

Plant Material and Culture Conditions: Shoot tips culture was established in *in vitro* from field grown plants of cultivar Grande Naine as described by Hamza [26]. The *in vitro* shoots cultures were cultured on MS [27] medium supplemented with 5mg/l BA (N^6benzyladinine) and 30 g/l sucrose. The medium was adjusted to pH 5.8 and prior to solidified by 0.6% phyto-agar, then, it was

steam sterilized in an autoclave under pressure of 1.2 Kg/cm^2 (121°C) over a period of 20 minutes. The cultures were incubated at a temperature of 25±2°C and 70-80% relative humidity with 16 h photoperiod and light intensity 1000 lux. These shoot tips were cut one time horizontally in such a way to keep the base of the explants intact and recultured on solid multiplication medium (MS medium supplemented with 5 mg/l BA). The vegetative apices were transferred to fresh medium three times at two weeks intervals to avoid phenol accumulation surrounding the cultured explants. When shoots multiplication began to form and continued growth warranted, they were subdivided into single shoots and subcultured on fresh multiplication media as described by Hassanein *et al*. [28]. Microshoots of 4-5mm, resulted from first subculture, were used as explants source for obtaining synthetic seeds.

Encapsulation Procedure: The most appropriate method for the preparation of synthetic seeds was the hydro-gel encapsulation method which described by Redenbaugh *et al*. [11]. In this method, sodium alginate of different concentrations (2 to 5%) was prepared by mixing with calcium free liquid MS medium and then the explants were mixed with the solution and dropped into the calcium chloride solution, where the ion exchange reaction occurs and sodium ions were replaced by calcium ions forming calcium alginate beads.

Effect of Different Concentrations of Sodium Alginate and Ca $Cl_2.2H_2O$ on Synthetic Seeds Formation: Sodium alginate of different concentrations [2, 3, 4 and 5%(w/v)] was prepared by mixing with calcium free liquid MS medium, prior to steam sterilized in an autoclave under pressure of 1.2 Kg/cm^2 (121°C) over a period of 20 minutes and dropped into the various concentrations of autoclaved calcium chloride solution (25, 50, 75, 100 and 125 mM) with pipette for 10 min. Where, ions exchanges occur between Na^+ and Ca^{++} forming calcium-alginate polymer. The effect of different concentrations of both Na-alginate and $CaCl_2$. $2H_2O$ combinations on capsules shape and texture was investigated.

Effect of Different Concentrations of Both Sodium Alginate and Calcium Chloride on Synthetic Seeds Germination: Microshoots of banana Grande Naine were individually dipped for a few seconds into various concentrations of sodium alginate solutions. Single microshoot and alginate mixture was picked up by sterile pipette (5 mm internal diameter) and was dropped into a beaker contained 100 mM $CaCl_2.2H_2O$ solution. Beaker was manually shacked for 10 min, where the ion exchange reaction occurs and sodium ions were replaced by calcium ions forming synthetic seeds, then, synthetic seeds were picked up and washed three times with autoclaved distilled water and draying using a sterilized tissue. Subsequently, synthetic seeds were cultured or germinated in Petri dishes contained 15 ml MS basal medium and incubated in the same conditions of establishment. Period for initial germination and germinated seeds number and germination percent were observed after 4, 8, 12 and 16 weeks.

Effect of Different Exposure Times to Calcium Chloride on Synthetic Seeds Germination: Microshoots of banana Grande Naine were individually dipped for a few seconds into 4% sodium alginate solutions. Then, the mixture of microshoot and Na-alginate was sucked and dropped into 100mM $CaCl_2.2H_2O$ and manually shacked for various exposure times (5,10 and 15 min) to Calcium chloride. Period for initial germination and germinated seeds number and germination percent were recorded after 4, 8, 12 and 16 weeks.

Effect of Different Concentrations of Benzyl Adenine (BA) on Synthetic Seeds Germination*:* Different concentrations of BA (0, 2.5 and 5.0 mg/l) were added to Na-alginate mixture. Microshoots were dropped in different mixtures of Na-alginate and were sucked and dropped into 100 mM $CaCl_2.2H_2O$ for ten minutes. Effect of adding BA to the Na- alginate on number of germinated seeds, germination percent, shoot number/synseed, shoot length (cm), growth vigor (was measured as described by Pottino [29]), root formation percent and roots number/ synseed were observed after 20 weeks.

Effect of Synthetic Seeds Composition on Germination and Growth Parameters of Banana Grande Naine Synthetic Seeds: Sodium alginate (4%) was dissolved in one of the following: water, MS, MS+ activated charcoal (AC) or MS+ AC+ 2.5 mg/l BA. Banana synthetic seeds formed as previously described, then it were cultured in culture jars contained 30ml MS basal medium. The effect of various components of Na-alginate mixture on synthetic seeds germinations and growth parameters of banana Grande Naine were observed after 20 weeks.

Table 1: List of RAPD and ISSR primers name and sequences.

	RAPD primers			ISSR primers	
NO.	Name	sequences	NO.	Name	sequences
1	OP-A01	CAGGCCCTTC	1	OP-A8	AGCAGCAGCAGCGC
2	OP-A12	TCGGCGATAG	2	OP-A9	AGCAGCAGCAGCAC
3	OP-A18	AGGTGACCG T	3	OP-A10	GCTGCTGCTGCTC
4	OP-A17	GACCGCTTGT	4	OP-Amic2	GACGATAGATAGATAGATA
5	OP-B12	CCTTGACGCA	5	OP-Amic3	AGATAGATAGATAGATA
6	OP-N16	AAGCGACCTG	6	OP-Amic5	CGGCACACACACACACA
7	OP-S253	GGCTGGTTCC	7	OP-Amic6	GGCCACACACACACACA
8	OP-S147	AGCTGCAGCC	8	OP-Amic7	CGACGACAGCAGCAGCAG
9	OP-S227	GAAGCCCAGC	9	OP-Mic7	CCTACCTACCTACCT
10	OP-S238	TGGTGGCGTT	10	OP-Mic8	CGACGACGACGACGA

Incubation Conditions: For germination, the synthetic seeds were maintained under the culture room conditions of light intensity (1000 lux), temperature at 25 ±2°C and 70-80% relative humidity with 16/8h day/night photoperiod.

Statistical Analysis of Synthetic Seeds Trails: Each treatment contained five Petri dishes or culture jars as replicates. Petri dish replicate contained eleven synthetic seeds while jar replicates contained five synthetic seeds. All experiments were arranged in completely randomized design. Differences among the various treatments were compared using LSD test at 5% according to SAS Institute [30].

Genetic Stability of Synthetic Seeds: Leaves samples were collected for RAPD and ISSR analysis of both mother plant of banana cultivar Grande Naine and random collection of leaves from plantlets which resulted from synthetic seeds.

DNA Isolation: The leaves of two samples were grounded to a fine powder in liquid nitrogen and total genomic DNA was extracted from 200 mg of grounded fresh leaves according to Doyle and Doyle [31] with some modifications. DNA concentration was measured spectrophotometrically (Nano Drop 1000, USA) at 260 nm and DNA templates were diluted to 50 ng/µl.

PCR Amplification and Electrophoresis (RAPD and ISSR markers): RAPD and ISSR markers were used for determination genetic stability of synthetic seeds. Ten RAPD primers (OP-A01, OP-A12, OP-A17, OP-A18, OP-B12, OP-N16, OP-S147, OP-S227, OP-S238 and OP-S253) and ten ISSR primers (OP-A08, OP-A09, OP-A10, OP-Amic2, OP-Amic03OP-Amic05, OP-Amic06, OP-Amic07, OP-Mic07and OP-Mic08), obtained from Bio Basic Inc.(Table 1), were used in PCR reactions. Each PCR reaction contained 100 ng of DNA template, 200 µM of dNTPs, 0.4 µM of Operon primer, 5 µl of 10 X of PCR buffer with 1.5 mM of $MgCl_2$ and one unit of *Taq* DNA polymerase, in a final volume of 25 µl.

PCR Program: PCR program consisted of 94°C for 5 min. 35 cycles of 94°C for 1 min followed by 37°C for 1 min and 72°C for 3min., then one cycle at 72°C for 7 min and at last the hold temperature was of 4°C.

DNA Electrophoresis: PCR products were separated by electrophoresis (5 V/cm) in 1.5% agarose gels and stained with 0.5 mg/l of ethidium bromide. A photographic record was taken under UV transilliminator (using Gell documentation camera).

DNA Electrophoresis Data Analysis: Each DNA amplified fragment was scored as present (1) or absent (0) fragment. Total amplified fragments were determined and polymorphic percent was calculated.

RESULTS

Effect of Different Concentrations of Sodium Alginate and Ca $Cl_2.2H_2O$ on Synthetic Seeds Formation: Synthetic seeds formation was affected by both Na-alginate and $CaCl_2.2H_2O$ concentrations. Results Table 2 and Fig. 1a cleared that sodium alginate concentrations positively affected synthetic seeds shape and texture, the high concentration the more rounded shape and the hardest texture of synthetic seeds. Also, increasing $CaCl_2.2H_2O$ concentration in cations exchange solution enhanced synthetic seed shape and texture. The more rounded and good texture was obtained from 4% sodium alginate when it dropped in 100mM $CaCl_2.2H_2O$ for form polymers of calcium alginate.

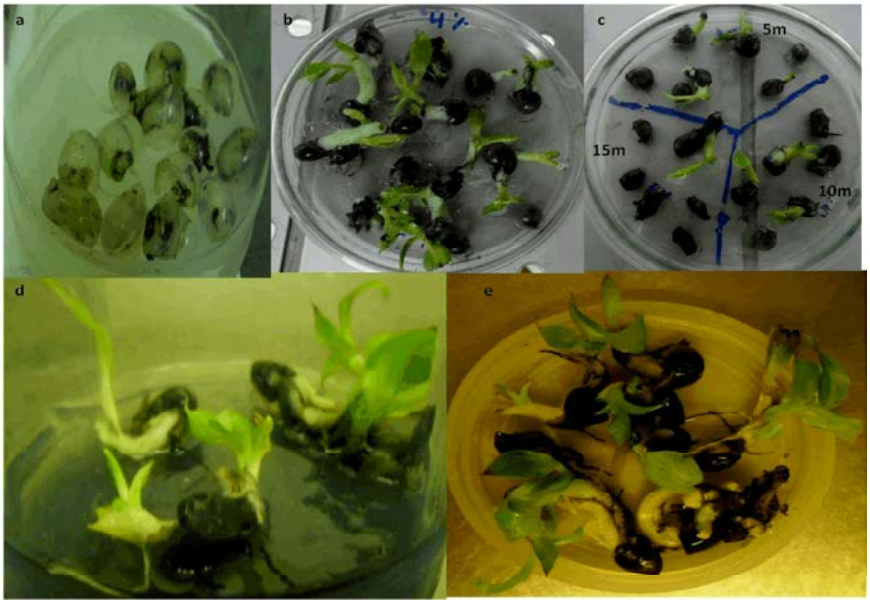

Fig. 1: Banana synthetic seeds formation and germination
a) Synthetic seeds formation at 4% Na-alginate and 100 mM $CaCl_2.2H_2O$
b) Germination of synthetic seeds resulted from 4% Na-alginate and 100mM $CaCl_2.2H_2O$ after 16 weeks
c) Germination of synthetic seeds resulted from different exposure times to 100mM $CaCl_2.2H_2O$ after 16 weeks
d) and e) Germination of synthetic seeds resulted from Na-alginate +2.5mg/l BA+ 1 g/l AC after 16 weeks.

Table 2: Effect of different concentrations of sodium alginate and Ca $Cl_2.2H_2O$ on synthetic seeds formation

$CaCl_2.$	2%Na-alginate		3% Na-alginate		4% Na-alginate		5% Na-alginate	
$2H_2O$ (mM)	Shape	Texture	Shape	Texture	Shape	Texture	Shape	Texture
25	Fragile	Very soft	Fragile	Very soft	Fragile	Very soft	Fragile	Very soft
50	Fragile	Very soft	Round with tall tail	Soft	Rounded with tall tail	Soft	Rounded with tail	Soft
75	Rounded with tall tail	Very soft	Rounded with tail	Soft	Rounded with shot tail	Soft	Rounded	good
100	Rounded with tail	Soft	Rounded with tail	good	Rounded	good	Rounded	Hard

Also, synthetic seeds were rounded and hard when it resulted from 5% sodium alginate dropped in both 75 and 100 mM $CaCl_2.2H_2O$.

Effect of Different Concentrations of Both Sodium Alginate and Calcium Chloride on Banana Synthetic Seeds Germination: Concerning the effect of sodium alginate concentration on period required for initial germination of banana cultivar Grande Naine synthetic seeds, results in Table 3 and Fig. 1b revealed that the period required for initiated synthetic seeds germination increased with increasing sodium alginate concentrations. Anyway, period required for initiated germination ranged from 21 days, when sodium alginate concentration was 2%, to 70 days, when sodium alginate was 5%. Sodium alginate at 2% concentration resulted in 20% germination after four weeks and reached to 100% synthetic seeds germination after twelve weeks. While, 3% and 4% sodium alginate gave 10% germination of synthetic seeds after eight weeks and reached to 100 and 90% synthetic seeds germination after 16 weeks. Sodium alginate at concentration 5% cleared the latest germination of synthetic seeds and the lowest germination percent after 16 weeks.

Effect of Different Exposure Times to Calcium Chloride on Synthetic Seeds Germination: Results in Table 4 and Fig. 1c cleared that there were positive relationship between exposure times to calcium chloride (cation exchange period) and period required for initiating synthetic seeds germination of banana cultivar Grande Naine. While there were negative relationship between exposure time to calcium chloride and synthetic seeds germination percent. Exposure times to $CaCl_2.2H_2O$ affected synthetic seeds germination number and percent in different way, after four weeks, only five minute

Table 3: Effect of different concentrations of both sodium alginate and calcium chloride on banana synthetic seeds germination.

		Germination after 4 weeks		Germination after 8 weeks		Germination after 12 weeks		Germination after 16 weeks	
Na-alginate	Period for initial germination (day)	NO	%	NO	%	NO	%	NO	%
2%	21	2	20	6	60	10	100	10	100
3%	42	0	0	1	10	6	60	10	100
4%	56	0	0	1	10	2	20	9	90
5%	70	0	0	0	0	1	10	2	20
LSD 0.0 5	12.7	0.5	--	3.9	--	3.1	--	3.7	--

Table 4: Effect of different exposure times to calcium chloride on banana synthetic seeds germination

		Germination after 4 weeks		Germination after 8 weeks		Germination after 12 weeks		Germination after 16 weeks	
Exposure time to 100 mM $CaCl_2.2H_2O$	Period for initial germination (day)	No	%	No	%	No	%	No	%
5 min	15	1	14.3	4	57.1	7	100	7	100
10 min	42	0	0	2	28.6	6	85.7	7	100
15 min	60	0	0	0	0	2	28.6	3	42.9
LSD 0.0 5	5.7	0.2	--	0.7	--	1.3	--	1.9	--

Table 5: Effect of different concentrations of benzyl adenine (BA) on banana synthetic seeds germination and growth parameters

BA conc. (mg/l)	No. of germinated seeds	Germination%	Shoots No. /synseed	Shoots length (cm)	*Growth vigor	Root formation%	Roots No. /synseed
0.0	5.60	80	1.0	5	3	50	3
2.5	6.30	90	3.1	4	4	30	1
5.0	7.00	100	3.5	3	3	10	0.5
LSD 0.0 5	1.32	--	1.3	0.87	0.84	5.9	0.4

*Growth vigor as described by Pottino [29], 2=slight growth 3= moderate growth 4= good growth 5= very good growth

exposure time resulted in 14% synthetic seeds germination and the same exposure time reached to 100% synthetic seeds germination after 12 weeks. Exposure to $CaCl_2.2H_2O$ for 10 minutes beginning with 28.6% germination after eight weeks and possessed the highest germination percent (100%) after 16 weeks. while exposure to $CaCl_2.2H_2O$ for 15 minutes resulted in 28.6% synthetic germination after 12 weeks and reached to 42.9% synthetic seeds germination after 16 weeks.

Effect of Different Concentrations of Benzyl Adenine (BA) on Banana Synthetic Seeds Germination: Results in Table 5 indicated that adding different concentrations of BA (0.0, 2.5 and 5.0 mg/l) to 4% sodium alginate solutions slightly affected number and percentage of synthetic seeds germination after twenty weeks. Germination increased with increasing BA in sodium alginate matrix from 80% synthetic seeds germination at control (free BA) to 100% synthetic seeds germination at 5.0 mg/l BA. Also, shoots number per synseed showed positive results with increasing BA concentrations in sodium alginate matrix. While, adding BA to the synseed matrix negatively affected shoots length. Growth vigor was good at 2.5mg/l BA. Root formation% and roots number per synseed were reduced with increasing BA concentration in synseed matrix.

Effect of Synthetic Seeds Composition on Germination and Growth Parameters of Banana Grande Naine Synthetic Seeds: Capsule components affected germination number and percent, the highest number and percent of germination resulted from sodium alginate dissolved in MS medium supplemented with activated charcoal and 2.5mg/l BA (6.8 and 97.1%, respectively), while, the lowest number and percent of germination resulted from sodium alginate dissolved in water. Shoots number per capsule, shoot length and growth vigor possessed the highest values when capsules resulted from sodium alginate dissolved in MS supplemented with activated charcoal and 2.5 mg/l BA (3.6 shoot/capsule, 4.5 cm and 4.5 for growth vigor, respectively). Finally, results revealed that adding AC and BA to MS medium before dissolving sodium alginate enhanced banana synthetic seeds germination, growth proliferation and growth parameters after germination as shown in Table 6 and Fig.1d,e.

Genetic stability of banana synthetic seeds: Genetic comparison between banana Grande Naine cultivar mother plant and resulted plantlets after short- time conservation and germination of synthetic seeds was carried out using two molecular markers, *i.e*. RAPD and ISSR (Table 7 and Fig. 2). Analysis of gel electrophoresis of ten primers of

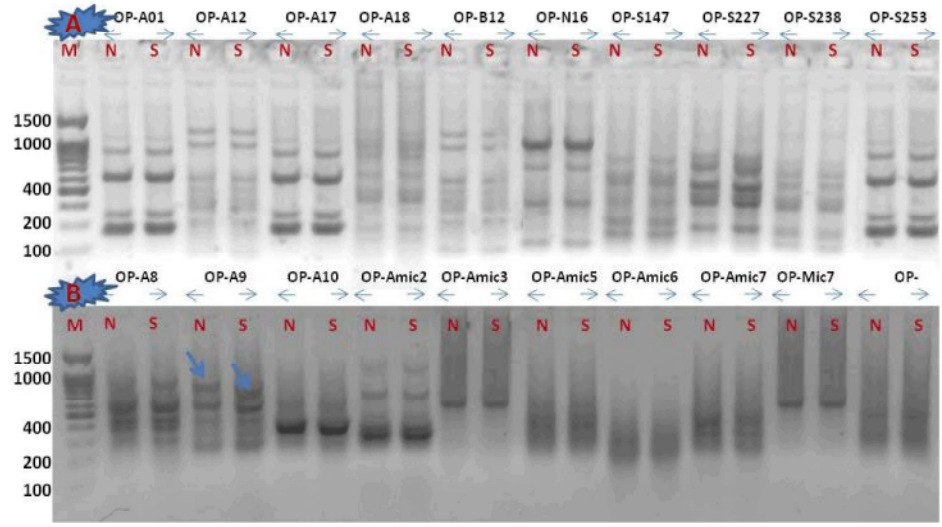

Fig. 2: Gel electrophoreses.
A: RAPD and B: ISSR molecular markers of mother plant and synthetic seeds of banana cultivar Grande Naine.
Where: N=mother plant S=synthetic seeds

Table 6: Effect of synthetic seeds composition on germination and growth parameters of banana Grande Naine synthetic seeds

	Synthetic seeds germination				
Treatments	(No)	(%)	Shoots number/capsule	Shoots length (cm)	*Growth vigor
Water	5	71.4	1.0	5.0	2
MS	6	85.7	1.0	5.3	3
MS+AC	6.5	92.9	1.0	6.2	5
MS+AC+2.5 mg/l BA	6.8	97.1	3.6	4.5	4..5
LSD 0.0 5	0.85	--	1.4	0.95	1.2

*Growth vigor as described by Pottino 1981, 2=slight growth 3= moderate growth 4= good growth 5= very good growth.

Table 7: Determination of synthetic seeds stability of banana cultivar Grande Naine based on RAPD and ISSR molecular markers

	RAPD marker			ISSR marker			
	Number of amplified fragments				Number of amplified fragments		
Primer	N	S	Poplymorfic%	Primer	N	S	Poplymorfic%
OP-A01	6	6	0	OP-A8	5	5	0
OP-A12	5	5	0	OP-A9	4	4	50
OP-A18	6	6	0	OP-A10	3	3	0
OP-A17	5	5	0	OP-Amic2	3	3	0
OP-B12	6	6	0	OP-Amic3	1	1	0
OP-N16	4	4	0	OP-Amic5	2	2	0
OP-S253	4	4	0	OP-Amic6	3	3	0
OP-S147	4	4	0	OP-Amic7	3	3	0
OP-S227	6	6	0	OP-Mic7	1	1	0
OP-S238	5	5	0	OP-Mic8	3	3	0
Total	51	51	0	--	28	28	0.071

Where: N=mother plant S=synthetic seeds

PCR-RAPD products cleared that there were 51 amplified fragments for both mother plant and proliferated synthetic seeds; all of them were monomorphic with zero percentage of polymorphism and total similarity. Also analysis of gel electrophoresis of ten primers of PCR-ISSR products showed 28 amplified fragments. Only one primer (OP-A9) gave two polymorphic fragments (50% polymorphism). The average total polymorphic percent was 0.071%.

From genetic analysis results, it could be concluded that synthetic seeds technique is a save method for both banana propagation and conservation which will produced true to type plants and the expected percentage of somaclonal variation is so low.

DISCUSSION

Obtained results cleared that synthetic seeds formation and texture depended on both Na-alginate and $CaCl_2.2H_2O$ concentrations, the more rounded and good texture was obtained from 4% sodium alginate when it dropped in 100 mM $CaCl_2.2H_2O$. Also, 5% sodium alginate gave the same results when it exposure to 75 and 100mM $CaCl_2.2H_2O$ but it is less economic and produced harder capsules. Results came in line with Cartes *et al*. [20] who reported that the hardness or rigidity of the beads mainly depends on the number of sodium ions exchanged with calcium ions. Banana cultivar Grande Naine synthetic seeds germination was affected by sodium alginate concentration, lowest concentration (2% sodium alginate) allowed synthetic seeds to germinate quickly, synthetic seeds initiated germination after four weeks and reached to100% germination after 12 weeks, this result may be due to the soft texture and bad formed capsules. While, high concentration of sodium alginate (4%) delayed banana synthetic seeds germination which initiated germination after eight weeks and reached to 90% germination after 16 weeks, this treatment may be suitable for short term conservation of banana and synthetic seeds delivery between laboratories. Formed encapsulated seeds from 5% sodium alginate resulted in synthetic seeds initiated germination after 12 weeks and reached to 20% germination after 16 weeks and need external investigation to study its ability for germination with long term conservation of banana synthetic seeds. Also, exposure times to $CaCl_2.2H_2O$ affected synthetic seeds germination, exposure for ten minutes to $CaCl_2.2H_2O$ resulted in 100% germination after 16 weeks. These results evidence that banana synthetic seeds germination was affected by sodium alginate and $CaCl_2.2H_2O$ concentrations and exposure times to $CaCl_2.2H_2O$ which allow to ions exchange and formation of polymerization, the responsible of capsule shape and texture which mainly affected germination, also the endogenous composition of the encapsulated plant may be affected the synthetic seeds germination. Results agree with Winkelmann *et al*. [12], Gill *et al*. [13], Ipekci and Gozukirmizi [14] and Standardi [32] who reported that hardening of calcium alginate bead is affected by the concentration of sodium alginate and calcium chloride and it may vary also in relation to the complexation time. Usually, higher texture corresponds to good protection during transport and manipulation, but higher difficulty to break the coating by the explant which leads to delay germination of synthetic seeds. Differently, *Geodorum densiflorum* required encapsulation in 4% Na-alginate and bead hardening in 50 mM $CaCl_2.2H_2O$ (15-20 min) for maximum conversion to plantlets (88%) as proved by Datta *et al*. [33].

Results cleared that banana synthetic seeds germination increased with increasing BA in sodium alginate matrix, from 80% synthetic seeds germination at control to 100% synthetic seeds germination at 5.0 mg/l BA. Adding 2.5mg/l BA, 1.0 g/l AC and MS medium to prepared sodium alginate matrix enhanced banana synthetic seeds germination compared with distilled water, MS or MS+2.5mg/l BA (97.1, 71.4, 85.7 and 92.9% of synthetic seeds germination, respectively). Also, growth proliferation and growth parameters after germination were affected by synthetic seeds composition. These results may be due to the ability of deliver the nutrients and the growth regulators to encapsulated banana microshoots to be able to synthetic seeds germination.

Finally, synthetic seeds germination varied according to capsulation matrix composition and nature of encapsulated plant. Results came in line with those conducted by Winkelmann *et al*. [12], Ganapathi *et al*. [17], Kumar *et al.* [34], Awal *et al*. [35] and Siong *et al*. [36] who suggested that the successful germination of the beades was probably due to the ability of microshoots to absorb the nutrients and the growth regulators in calcium alginate beads. Synthetic seeds of rice with artificial endosperm constituents of MS nutrient, sucrose (3%w/v), 0.5 mg/l IAA, 0.5 mg/l NAA, 0.5 mg/l BA and activated charcoal (1.25%w/v) gave maximum germination rate of 30% by using somatic embryos at globular stage as propagules.in other study, isolated microshoots of *Brassica oleracea* var. botrytis (cauliflower) encapsulated in MS supplemented with 0.3 mg/l NAA and 3.0 mg/l BA gave high germination percentage. They also reported that the inclusion of activated charcoal had enhanced the germination to the maximum extent by increase the diffussion of gases, nutrients and respiration of embryoids. Results of genetic analysis of banana synthetic seeds using RAPD and ISSR genetic markers revealed that synthetic seeds is a save method to produce true to type plantlets. The same results were conducted by Nyende *et al*. [24] who reported that there was potential advantage of synthetic seeds for genetically identical to natural plants. Also, Gangopadhyay *et al*. [25]

stated that the genetic stability of plantlets derived from encapsulated *Ananas comosus* microshoots was proved by random amplified polymorphic DNA (RAPD) and ISSR techniques.

CONCLUSION

Artificial seed production is an outstanding technique used to propagate and preserve plants and has been applied on many plants. However, only a few has been reported on researches on growth and the second production [37], so, production of banana artificial seeds and test its ability on conservation and regeneration after short term conservation is an important goal. Banana synthetic seeds formation and germination were affected by sodium alginate concentration, calcium chloride concentration, exposure times to calcium chloride and composition of synthetic seeds. The optimized protocol for producing banana synthetic seeds is 4% sodium alginate dropped in 100 mM $CaCl_2.2H_2O$ for 10 min. This protocol resulted in banana synthetic seeds germination after short time conservation (16 to 20 weeks). Synthetic seeds composition had a noticeable effect on germination and proliferation of banana synthetic seeds, the optimum composition was 4% sodium alginate dissolved in MS medium supplemented with 2.5 mg/l BA, 1g/l AC and 30 g/l sucrose. This composition allowed saving the synthetic seeds multiplication ability after short time conservation. The produced banana synthetic seeds were stable genetically based on RAPD and ISSR molecular markers. This protocol may be useful in low production cost, short time conservation, ease of handling and ease of exchange of plant materials between different laboratories in different counties. Finally, it must be pointed that synthetic seeds composition may be differed depending on plant type, where its endogenous construction may affected its response.

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