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RESEARCH ARTICLE

Development of Artificial Seed Technology and Preservation in Sugar Beet

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Abstract Germplasm can be effectively stored in the form of synthetic seeds that offer several advantages, easy handling, storability, reduced size of propagules, and transportability. Shoot tips obtained from in vitro seedlings cultures of sugar beet (*Beta vulgaris*) were encapsulated in 4 % sodium alginate. For multiplication of sugar beet propagules, the medium contained 2 mg l^{-1} BA gave the highest number of shoots and leaves. However, the best results of shoot length were observed with 2 mg l^{-1} kin. The addition of MS medium to alginate capsules, with 30 g l^{-1} sucrose, significantly improved their germination rate than 40, 50 and 60 g l^{-1}. In vitro storage of capsules was also evaluated by supplementing osmotic agents, mannitol, or sorbitol to the media. Adding sorbitol or mannitol to the media at 0.05 M increased plantlet survival. Francesca genotype was better suited for encapsulation than the Toro genotype. Among different types of auxins used for in vitro root formation, 2 mg l^{-1} of NAA with aeration were more effective than other treatments. The molecular studies using RAPD technique with eight primers proved that, the same dose of conservative material (mannitol or sorbitol) that were used to conserve the encapsulated plants. After the re-growth from plantlets it showed that adding mannitol or sorbitol was not only the factor but interaction between the conservative materials and the genotype of the plants also play an important role in the conservation.

Keywords Mannitol · Sorbitol · Sugar beet · Synthetic seeds · RAPD

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Introduction

Sugar beet (*Beta vulgaris* L.) is the most important sugar producing crop in Europe and it supplies about 40 % of the world total sugar production (Atanassov 1986). Breeding of sugar beet is mainly carried out conventionally but in the past couple of decades, the use of molecular techniques, more specifically genetic transformation technologies, has drastically increased (Ivic et al. 2001). The development of an efficient protocol for plant regeneration from cultured explants is a prerequisite for the genetic manipulation and improvement studies. Sugar beet is known to be a recalcitrant species with respect to in vitro culture and genetic transformation (Elliott et al. 1996; Krens et al. 1996). Adventitious shoot regeneration from several cultured explants has been often employed for the propagation of elite genotypes of sugar beet (Detrez et al. 1988; Ritchie et al. 1989; Zhong et al. 1993; Grieve et al. 1997) but there was a high degree of variability in the regeneration frequencies from various explants of different genotypes (Jacq et al. 1992; Saunders and Tsai 1999). Distribution and exchange from field genebank is difficult because of the vegetative nature of the material and the greater risks of disease transfer. In vitro techniques are of great interest for the collecting, multiplication and storage of plant

germplasm (Engelmann 1991). Moreover, establishment of synthetic seeds have multiple advantage including ease of handling, potential long-term storage and low cost of production and subsequent propagation (Ghosh and Sen 1994). RAPD technology has been successfully used for measuring genetic diversity within and between in plants. Patterns of variation observed are shown to closely resemble those obtained using more morphological characters (Howell et al. 1994; Virk et al. 1995). The present study aims to develop an efficient protocol for in vitro storage of sugar beet capsules and carry out molecular studies using RAPD technique for the re-growth plantlets after capsulation to compare the effect of two kinds of sugar alcohol (mannitol and sorbitol) on the conservation of the genetic material.

Materials and Methods

Establishment of Explants and Multiplication

Seeds of two sugar beet breeding lines *Francesca* and *Toro* were treated with 70 % (v/v) alcohol for 5 min, and sterilized with 7.5 % (v/v) sodium hypochloride for 1 h in the presence of 0.5 ml Tween 20 per 100 ml solution. Seeds were then rinsed several times with sterile distilled water and left in sterile distilled water for 16–20 h. After sterilization, seeds were cultured on MS medium (Murashige and Skoog 1962) containing 3 % (w/v) sucrose, 8 g l^{-1} agar, 0.5 mg l^{-1} 2,3,5-triiodobenzoic acid (TIBA) and 1.0 mg l^{-1} 6-benzylaminopurine (BA). The pH was adjusted to 5.8 (Gürel et al. 2003). After 30 days, the shoot tips were transferred to hormone-free MS medium for further multiplication, so as to allow the shoots to attain a height of 3.0 cm. Finally, about 0.2–0.5 cm long segments comprising defoliated single shoots were taken as explants after two sub-cultures of 4 weeks each. For shoot regeneration, shoots obtained from the above experiment were transferred to MS medium containing 0.0, 1.0, 1.5 and 2.0 mg l^{-1} BA, Kinetin (Kin) or Thidiazuron (N-phenyl-N′-1,2,3-thiadiazol-5yl-urea (TDZ).

Influence of Ventilation Closure on Root Development of Two Sugar Beet Genotypes

Individual shoots were cultured on root induction MS medium containing indole-3-butyric acid (IBA) or a -naphthalene acetic acid (NAA) at concentrations, 0.0, 1.0, 2.0 and 3.0 mg l^{-1}. They were further incubated for root development, under aerated and non-aerated conditions, in glass jars (350 ml) containing 60 ml medium for 1 month. For aeration treatments, the glass jars were sealed with a Sun Cap closure (0.02 m filter) which allowed gases to diffuse in and out of the jar. Polypropylene (PP) membrane seals were used for non-aerated controls. Ten replicates per treatment with 10 explants in each were used for every experiment which was repeated twice. All cultures were maintained at laboratory conditions (25 °C with a 16 h light photoperiod of 2,000 lux). The plantlets were further placed in a temperature controlled (28 °C) greenhouse for 2 months.

Storage of Germplasm

Effect of Different Sucrose Concentration on Capsules Re-Growth

Individual shoot tip explants were transferred into 4 % of sodium alginate in liquid MS medium supplemented with 30, 40, 50 or 60 g l^{-1} sucrose. Drops of sodium alginate solution containing one shoot tip each explants were then slowly dropped into 100 mM Ca $(NO_3)_2{\cdot}4H_2O$ solution and stirred continuously for 30 min on a shaker. Finally, the artificial seeds were cultivated in a germination medium in jars (250 ml) on MS medium supplemented with 0.25 mg l^{-1} BA and 2.5 g l^{-1} agarose. They were then left in the culture chamber at a temperature of 20 °C in complete darkness. Data were taken after 6 weeks.

Effect of Sugar Alcohol (Mannitol or Sorbitol) on Capsules Re-Growth

Attempts in the in vitro storage of synthetic seeds have focused on imposing some sort of stress on the cultures in order to achieve reduced growth. One means of growth limitation has been achieved by mannitol or sorbitol. Since the germplasm remains viable, it can be grown again into shoots. Sodium alginate (4 %) was prepared using MS liquid medium and 100 mM of Ca $(NO_3)_2{\cdot}4H_2O$ solution for complexation and autoclaved. Encapsulation was accomplished by mixing the shoot tip explants into the alginate and dropping these into the Ca $(NO_3)_2{\cdot}4H_2O$ solution. The droplets containing the explants were held for at least 30 min to achieve polymerization of the sodium alginate, beads were collected and rinsed with sterile distilled water to wash away Ca $(NO_3)_2{\cdot}4H_2O$ residues. Thereafter beads were cultured on MS medium supplemented with mannitol or sorbitol at 0.05, 0.1, 0.2, and 0.3 M. Finally, the artificial seeds were cultivated in a germination medium in 250 ml jars on MS medium supplemented with 2.5 g l^{-1} of agarose. They were then kept left in the culture chamber at a temperature of 20 °C in complete darkness for 6 weeks to slow growth. Finally, the artificial seeds were cultivated in a germination medium (for re-growth) in 250 ml jars on MS medium supplemented with 7 g l^{-1} agar for 2 months.

Randomly Amplified Polymorphic DNA

Re-growth shoots from synthetic seeds of the two cultivars of sugar beet (*Beta vulgaris*) were used; *Francesca, Toro*. Both cultivars were encapsulated and storage with two alcohol sugars; Mannitol or Sorbitol using the same concentration 0.05 mM. They compared with two capsulated samples of both cultivars (without mannitol or sorbitol) as a control.

Molecular Analysis

Random Amplified polymorphic DNA analysis (RAPD) was used to compare the effect of mannitol or sorbitol as conservative for genetic materials of the survival re-growthed plantlets from capsules, of controls and treatments with two alcohol sugars; Mannitol or Sorbitol, that contain the original and normal DNA.

DNA Isolation

The DNA were extracted from six samples of survival plantlets, they classified as follows: two controls; *Francesca* (FC) and *Toro* (TC), treated encapsulated samples with mannitol (0.05 M); *Francesca* (FM) and *Toro* (TM), and treated encapsulated samples with sorbitol (0.05 M); *Francesca* (FS) and *Toro* (TS). The DNA extraction was performed according to the protocol of Genomic DNA purification kit from "Fermentas" using 0.1 g from each fresh sample of shoot tips, then 2.0 µl from RNase "Fermentas" was added to digest the RNA.

Polymerase Chain Reaction (PCR)

Eight random primers were used to establish the RAPD analysis. These primers and their sequences were listed in Table 1. Each primer was used in a PCR reaction, The total amount of each reaction was 25 µl and consisted of: (1.25 U) 0.25 µl of Dream *Taq* DNA polymerase "Fermentas", 2.5 µl of Dream Taq buffer including $MgCl_2$, 0.4 µl of dNTPs (40 mM, 10 mM each) "Viogene", 2.0 µl of random primer (25 pmol) and 50 ng of genomic DNA. The PCR program was installed and carried out as follows; initial denaturation 94 °C/10 min followed by 40 cycles with 94 °C/50 s to denaturize the DNA, 37 °C/30 s to anneal the primer with the template DNA and 72 °C/30 s to extent the primer, and then the final extension at 72 °C/5 min. The PCR patterns were separated on 1.5 % agarose gel "Axygen" and were weighted with 100 bp DNA ladder "Fermentas", then the patterns were visualized and photographed on the UV transilluminator of the Gel documentation analysis system "Bio-RAD". The analysis of each gel was done using "Gel Analyzer 3 software", while "SPSS ver. 10 software" was used to calculate the similarity matrix and draw the phylogenetic tree.

Results and Discussion

Effect of Cytokinin Type (BA, Kin and TDZ) at Different Concentrations and Genotype (*Francesca* and *Toro*) on Growth and Development After 6 Weeks In Vitro

The effect of different BA, Kin and TDZ levels to proliferation medium was studied on subsequent shoot development from shoot tip explants of sugar beet (*Beta vulgaris* L.) (Fig. 6A). Explants were excised from starting medium containing 1, 1.5 or 2 mg l^{-1} for 5 weeks. Results of analysis of variance of the shoot production data are presented in Fig. 1. There was significant difference among various levels of BA and genotype. In other words, with increasing of BA concentration, the length of regenerated shoot was decreased, Mezei et al. (2006) regardless of starting explant and regeneration protocol used, this type of sugar beet regeneration and multiplication is strictly dependent on interaction of genotype and concentrations of phytohormones in nutrient medium. MS medium containing 2.0 mg l^{-1} BAP of different varieties of the same species or even sometimes different plants of the same variety (Gürel 1997), although some species, for instance sugar beet (*Beta vulgaris* L.), are recalcitrant to in vitro regeneration (Gürel and Wren 1995).

Effect of Sucrose Concentrations with Gel Matrix on Re-Growth Sugar Beet Capsules

In this study, 30, 40, 50, and 60 g l^{-1} sucrose and 4 % of sodium alginate were investigated as gel matrix for encapsulation of shoots of two genotypes of sugar beet. The results presented in Fig. 2 indicated that, increasing sucrose concentration from 30 to 60 g/l decreased percentage of conversion of encapsulation. At low concentration (30 g l^{-1}) of sucrose, capsules increased of strong conversion and shoots turned green (Fig. 6D). However, at the highest level (60 g l^{-1}) capsules were slowly conversion and decreased shoots proliferation. Results also markedly increased conversion of *Francesca* capsules than Toro without significant in

Table 1 List of primers and their sequences

No. of primer	Primer name	Sequence 5′–3′
1	OPC19	GTTGCCAGCC
2	OPA03	AGTCAGCCAC
3	OPX11	GGAGCCTCAG
4	OPC15	GACGGATCAG
5	OPW04	CAGAAGCGGA
6	OPX17	GACACGGACC
7	OPD13	GGGGTGACGA
8	OPT08	AACGGCGACA

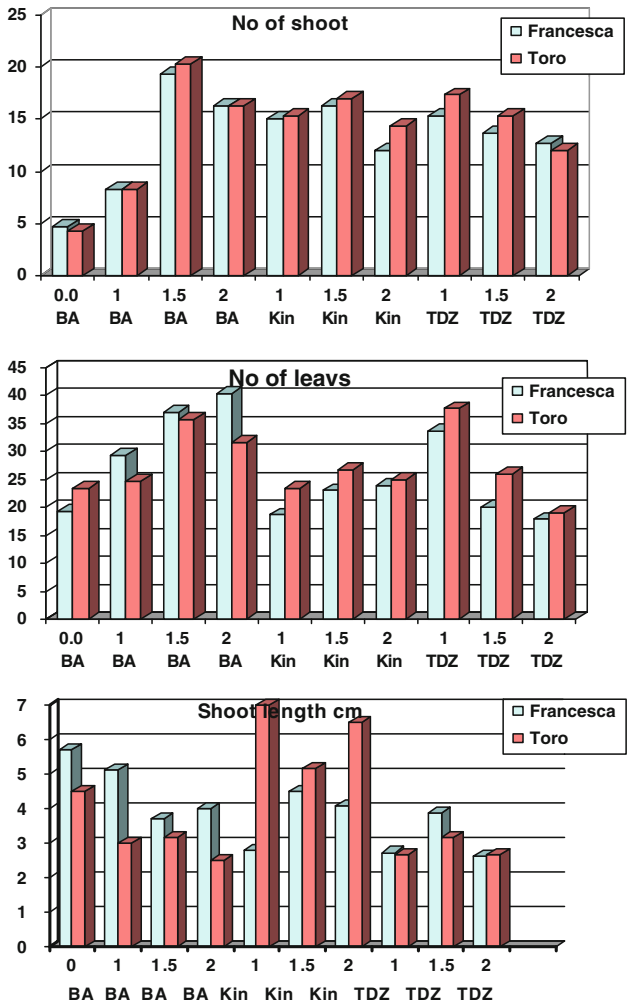

Fig. 1 Shoot development of (*Francesca* and *Toro*) at different concentrations of cytokinin

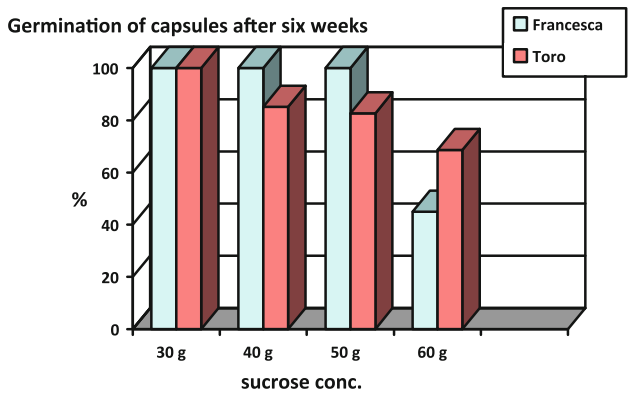

Fig. 2 Effect of sucrose concentrations with gel matrix on growth and development of sugar beet capsules

between. The presence of nutrients in gel matrix, which served as a nutrient bed around the encapsulated buds of sugar beet affected growth and survival. In this respect, Panis (1995) showed that sucrose is known to provide a carbon source for in

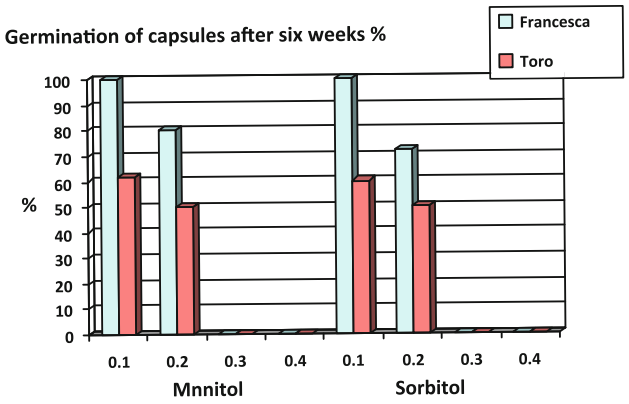

Fig. 3 Effect of different levels of mannitol or sorbitol on germination of synthetic seeds after 6 weeks

vitro propagules, and its inclusion in the alginate matrix enhanced plant recovery. (Huda et al. 2007) among the different carbon sources, sucrose showed better performance for both varieties of (*Solanum emelongena* L.) synthetic seeds.

Effects of Sugar Alcohol Concentrations and Genotype on Re-Growth and Development of *Francesca* and *Toro* Capsules After 6 Weeks

The addition of osmotic agents sorbitol or mannitol at 0.05 M to the media has increased culture survival, however, with deterioration of cultures in terms of quality of growth (Fig. 6E). A decline in survival and re-growth occurred on culture storage at higher concentrations of osmotic agents 0.2 or 0.3 M mannitol or sorbitol each (Fig. 3). The use of the osmotic agents was not an effective procedure. The most suitable to conversions of capsules was *Francesca* than *Toro* genotype. These results corroborate with Westcott (1981) descriptions on the toxic effects of mannitol in *Solanum* germplasm storage. The research in synthetic seed technology has been prolific, although with few applications on in vitro storage. However, Hemant et al. (2010) showed that in vitro storage of shoot cultures was also evaluated by supplementing osmotic agents, mannitol, or sorbitol to the media. Such treatment had a negative impact on post-storage re-growth (at 25 °C).

Effect of Auxin Concentrations and Aeration on Growth and Development of Sugar Beet Rooting In Vitro

Rooting of *Francesca* and *Toro* shoots is readily achieved when cultured on MS medium supplemented with 2.0 mg l^{-1} NAA (Fig. 6B, C). Auxins are well known to induce adventitious root formation in many plants when compared to the control explants. IBA at 1.5 or 2.0 mg l^{-1} resulted in the tallest plants and increased both leaves and roots number as shown in Figs. 4 and 5. Gürel and Wren

(1995) showed that in which a high NAA concentration (1–30 mg l^{-1}) was found to inhibit root growth after induction, whereas transferring leaf explants from high NAA to hormone free medium, after an initial incubation for 1–4 days at high NAA, greatly enhanced subsequent root growth. These results are in line with those reported by Gürel and Gürel (1996) reported that root induction was readily achieved within 2 weeks when shoots were transferred to MS medium supplemented with 2.0 mg/l naphthaleneacetic acid (NAA) and 2.0 mg l^{-1} silver nitrate ($AgNO_3$). Aeration was found to be most effective in producing roots. Using closures with filters or vented vessels, which allow gas exchange, increasing the photosynthetic capacity, rooting rate, and survival of plants after transfer to ex vitro conditions. This result is in agreement with (Lucchesini and Mensuali-Sodi 2004) found that, during rooting of *Phillyrea latifolia* L., the use of ventilated vessels in comparison with the closed ones enhanced development of roots, and doubled the dry weight of plantlets. Therefore, the main principle of acclimatization of sugar beet from in vitro culture is putting it under conditions where air humidity can be gradually lowered (Hazarika 2003). Medium aeration at the proximal end of the microcutting is more important than shoot orientation for in vitro rooting of lentil microcuttings (Newell et al. 2006). The rooted plants are transferred to pots and kept for 3 weeks in the growth chamber where humidity is gradually reduced. Plants are then placed on the room temperature for 1 month and then transferred to the greenhouse, where acclimatization lasts up to 2 months.

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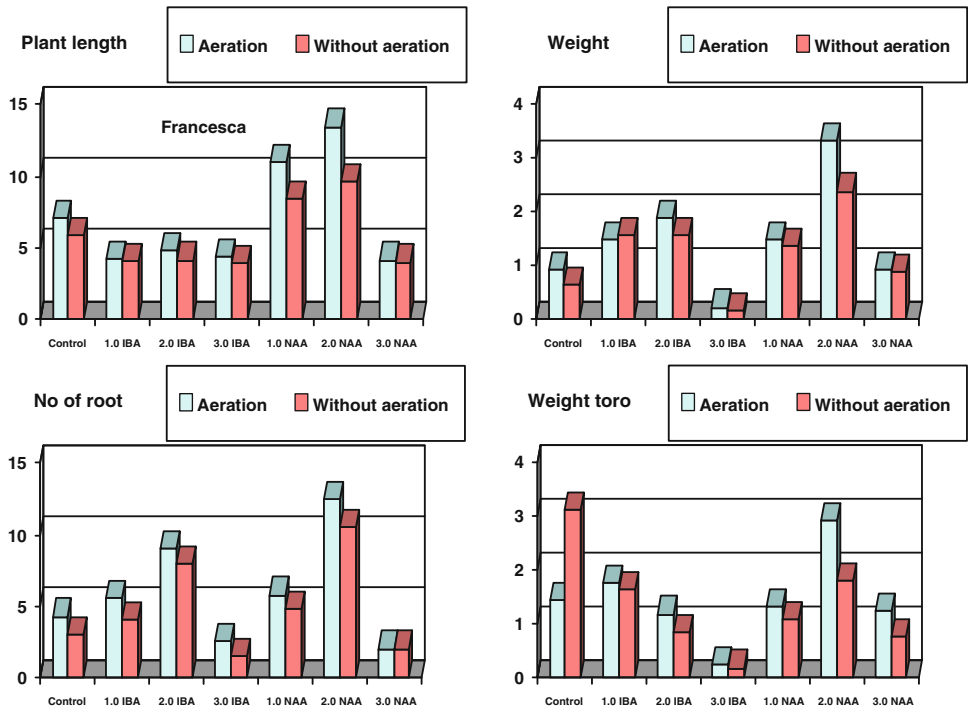

Fig. 4 Development of rooting of *Francesca* after 6 weeks in vitro

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RAPD Analysis

The present molecular study aimed to compare the effect of two kinds of alcohol sugar (mannitol or sorbitol) on the conservation of the genetic material of the encapsulated seed using RAPD technique. The use of RAPD primers offers the potential of acquiring more cost effective data than in the case with other technologies. The genetic analysis was performed using RAPD markers because this method does not require knowledge of the sequence of the DNA under study (Wolfe and Liston 1998). The most attractive feature of RAPD analysis is that it can be used on

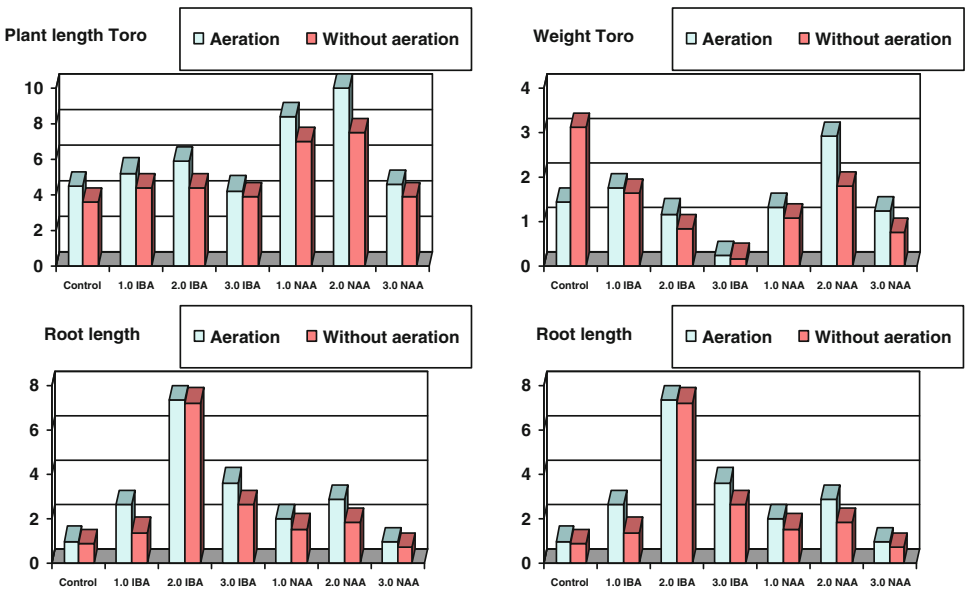

Fig. 5 Development of rooting of *Toro* after 6 weeks in vitro

Table 2 Numbers of detected monomorphic and unique bands, total number of bands (mono. + poly. + unique) and the % of polymorphism (polymorphic + unique/total no. of bands × 100) for all primers

Primer	Monomorphic bands	Unique bands			Total no. of bands	Polymorphism %
		No.	Sample	MW		
OPX11	0	2	TC	341.82	6	100
			TM	330.77		
OPX17	2	0	0	0	5	60
OPC15	0	2	TC	1010.7	5	100
			TC	302.6		
OPC19	1	2	FS	941.06	9	88.9
			FC	650.46		
OPW04	0	1	TM	312.33	7	100
OPA03	2	3	TC	1151.22	12	83.33
			TM	615.141		
			TS	602.927		

TC Toro with control, *TM* Toro with mannitol, *TS* Toro with sorbitol, *FC Francesca* with control, *FM Francesca* with mannitol, *FS Francesca* with sorbitol

pooled DNA samples to rapidly screen for linked DNA markers (Michelmore et al. 1991). These primer sequences vary in different varieties of sugarcane, and this variability may be used to develop molecular markers for mapping sugarcane genes and traits, where these sequences are the part of sugarcane genome predicted to be most immediately useful to plant breeder and geneticists. Furthermore, this information will be useful for developing future breeding programs (Tabasum et al. 2010). With the advent of molecular markers, it is now possible to make direct comparison of genetic diversity at the DNA level without some of the over simplifying assumptions associated with calculating genetic diversity based on pedigree history (Brar 2002). Primers OPD13 and OPT08 were failed to

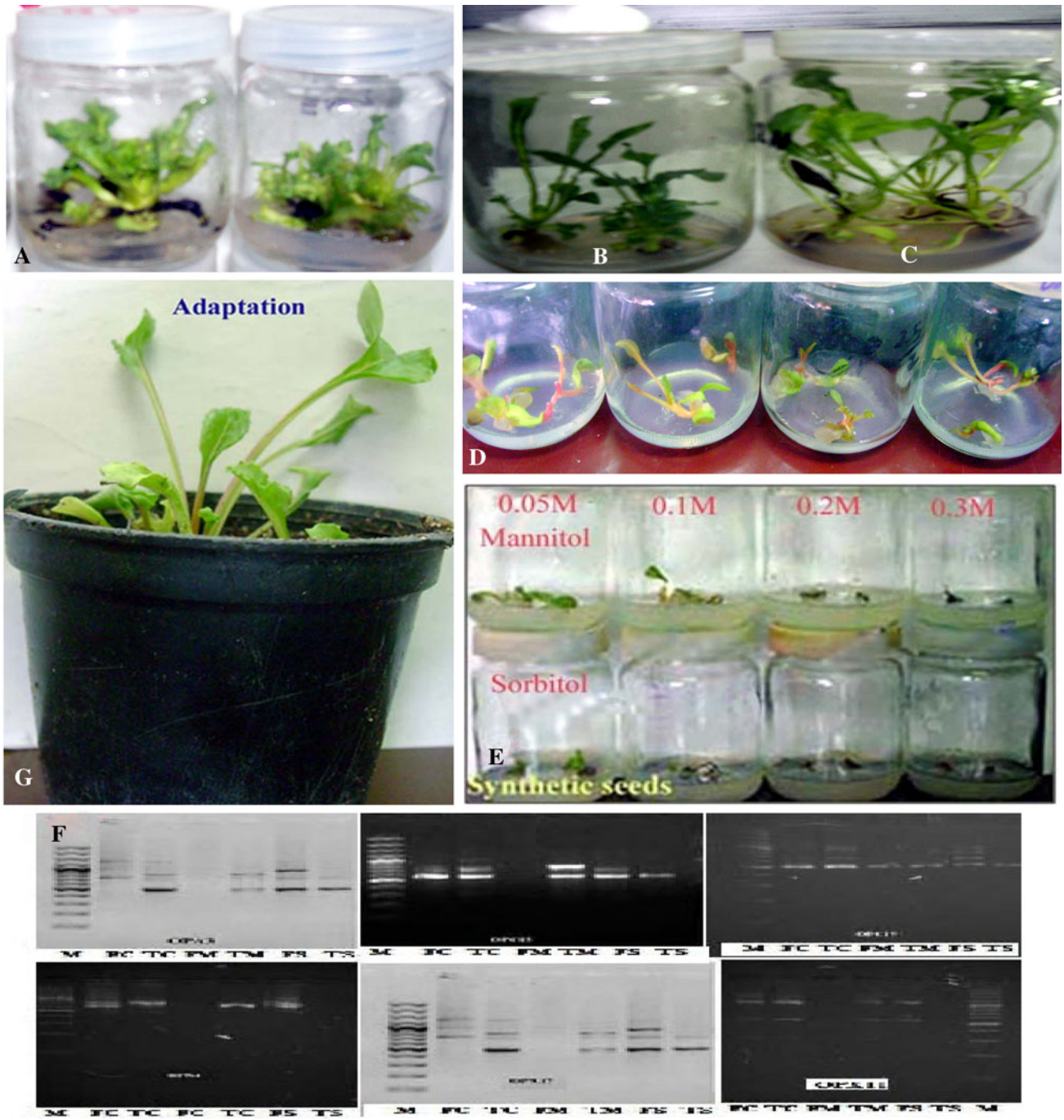

Fig. 6 Multiplication (**A**). Rooting of plantlets under aeration or without aeration (**B**, **C**). Encapsulation of shoot tips with 4 % sodium alginate and different sucrose concentrations (**D**). Germination of encapsulated after storage in mannitol or sorbitol medium for 30 days. (**E**). RAPD profile of capsules storage on mannitol or sorbitol (**F**)

reveal any amplified fragments. While the other six primers OPA03, OPC15, OPC19, OPW04, OPX17 and OPX11 success to detect 12, 5, 9, 7, 5 and 6 amplified fragments respectively. Also Maria et al. (2002) detect 11 amplified fragments when they used OPA03. The first observation in this study was the decreasing number of detected patterns arrange between zeros to eight amplified fragments of PCR products, which indicates to the ability of using this random method to detect specific molecular marker. The successful outcome of these random primers to detect the genetic polymorphism that appear among the control and treated samples with (mannitol or sorbitol) of the plantlets of sugar beet for each cultivar display in Table 2 and Fig. 6F. Primers OPW04, OPC15 and OPX11 scored the highest percentage of polymorphism 100 %, while OPX17 had 60 % polymorphism. Primer OPC19 only was revealed two positive markers (unique bands) with *Francesca* cultivar. On the other hand, the rest of primers were specified for *Toro* cultivar, the unique bands arranged from one to three, except primer OPX17 failed to detect any positive marker with both cultivars. The similarity matrix displayed in Table 3 according to all the tested random primers that: firstly; the similarity between the controls of two cultivars (TC and FC) was 76.0 %. Secondly; the treatment of mannitol to conserve the genetic material of the encapsulated explants was failed with *Francesca* (FM) comparative with Toro(TM) because the similarity between FM and FC was 30.3 % while between TM and TC was 65.8 %. In contrast, the treatment of sorbitol was successive with *Francesca* more than with Toro, displaying in 71.1 % similarity between FC and FS while between TC and TS was 51.3 %. The differences in response of two cultivars to the same treatment were rendered to 24 % of diversity between them. Thirdly; the similarity between both cultivars treated with mannitol was 62.1 %, and with sorbitol was 51.6 % while the similarity between the untreated samples was 76 %, that indicates to another observation which is the treatment with sorbitol decreased the similarity between the cultivars more than mannitol. In conclusion, to judge on the effect of sorbitol or mannitol to conserve the genetic materials in the encapsulated sugar beet it needs more studies because the analysis of data indicated that both of them increased the polymorphism. On the other side, treatment with mannitol was more suitable for *Toro* cultivar and treatment with sorbitol was more suitable for *Franssica*, in addition to mannitol was better than sorbitol to conserve the genetic material comparing with the control. And this results agree with the previous studies that found the high concentration of sorbitol induced biochemical changes, and this salt stress shock induce effects on the amino acids metabolism (Heng et al. 1999) (Fig. 7).

Table 3 Proximity matrix

Case	Matrix file input					
	FC	TC	FM	TM	FS	TS
FC	1.000					
TC	.760	1.000				
FM	.303	.486	1.000			
TM	.524	.652	.621	1.000		
FS	.711	.653	.438	.732	1.000	
TS	.514	.513	.455	.516	.529	1.000

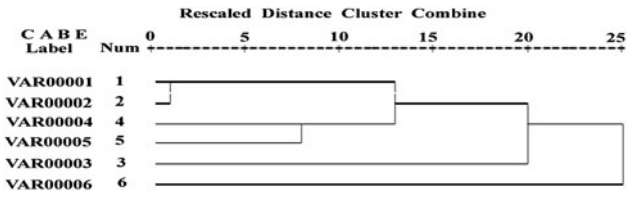

Fig. 7 Dendrogram using average linkage (between groups)

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