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Mid-term Storage and Genetic Stability of Strawberry Tissue Cultures

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Abstract: The objectives of the present investigation were to find out a medium-term *in vitro* preservation protocol of strawberry and molecular identification of the stored cultures. The medium term preservation study was initiated under 4 ºC and dark conditions using i*n vitro* grown leaflets cultures. Explants were subjected to different concentrations of mannitol and sorbitol (0.1, 0.2 and 0.4 M) as osmotic regulators added to culture medium with or devoid of Indolbutyric acid (IBA). Results show that number of shoots was increased when medium was supplemented with 1 mg IBA/l and 1 M sorbitol and preserved for 15 months. On the contrary, the highest percentage of healthy shoots (76 %) was noticed with medium contained 0.2 M sorbitol without IBA. Whereas the highest number of roots per shoot was occurred on the medium supplemented with 1 M sorbitol devoid of IBA. Also, sorbitol containing medium registered best results of plantlet recovery. Determination of stability was performed by assessment of randomly amplified polymorphic DNA (RAPD).The total number of the amplified RAPDs produced varied depending of primers used. The analysis of RAPD marker did not show any variation among the preserved and non-preserved material with the most primers used.

Key words: Strawberry, *in vitro* preservation, mannitol, sorbitol, RAPD analysis

INTRODUCTION

Preservation of plant genetic resources has become extremely important for crop improvement to face the increasing depletion of natural resources. The aseptic culture of plant cells and tissues under defined conditions in the laboratory is termed *in vitro* culture[25]. This technique can be used for the multiplication and storage of disease-free plant germplasm. *In vitro* conservation is used as complementary methods to field maintenance and involves the sustainment of explants in a pathogen-free environment and distribution of clonal material. It is widely used for the conservation of species which produce recalcitrant or no seed, and for vegetatively propagated plant material[6]. Application of *in vitro* techniques for germplasm conservation will mainly rely on the system's ability to regenerate a whole plant that will survive in the field and exhibit genetic stability over time[34]. Among the different methods of *in vitro* preservation is the short- and medium-term storage to increase the interval period between subcultures by reducing growth. This might be achieved by the use of modified environmental conditions[36], modified culture medium[35], growth retardants[12,18], osmotic regulators[32,7,24,19,19,38] and/or reduction of oxygen concentration[4]. Slow growth storage via *in vitro* cultures has been reported in many species[37]. Low temperature (2-10 ºC) for minimal growth storage of cultured plant cells and organs has been applied successfully to grape[20] and apple[16]. The addition of osmoticums or growth retardants to the medium has proved efficient for reducing growth rates of different plants species. Osmoticums such as manitol or sorbitol reduce mineral up take by cells through differences in osmotic pressures thereby retarding plant growth[5,30]. The application of DNA technology in agricultural research has progressed rapidly over the last twenty years, especially in the area of variety identification. More recently, molecular marker techniques using Polymerase Chain Reaction (PCR) based technique has become increasingly popular for fingerprinting and varieties identification[22,29]. Random Amplified Polymorphic DNA (RAPD) markers, utilizing PCR amplification from single arbitrary primers, were developed by Williams and his co-workers[33]. Dominant RAPD-markers have been used for the identification of different plant species, as well as for assessing genetic diversity[10,15,21].

Strawberry (vegetative propagated plant) is stored as *in vitro* plantlets for plant breeding, as virus-free planting stock, and for germplasm preservation[23]. The most widely applied technique is temperature reduction, combined with a reduction in the

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concentration of nutritive elements or decrease in light intensity or storage in the dark. In this respect, high survival rates of strawberry plantlets was obtained from successfull storing at 4°C in the dark for 12 and 24 months[25,26]. The present study aims to develop a procedure for *in vitro* mid-term storage of strawberry by investigate the effect of addition of sorbitol and manitol to culture medium and incubation at low temperature and determine the genetic stability of stored cultures using RAPD analysis.

MATERIALS AND METHODS

Plant Material and Tissue Culture: Strawberry variety (*Fragaria x ananassa*) named Camarosa was used. Runners of 2 to 3 cm in length were surface sterilized by 70% ethanol for 1min followed by 20% sodium hypochlorite solution (commercial) for 20 min and then rinsed three times with sterile distiller water. Tissue cultures were initiated from shoot tips consisting of meristems plus 2 or 3 leaf primordial which were excised and cultured onto glass tubes (100 x 25 mm) contained Knop's medium supplemented with benzylamino purine (BAP) 1.0 mg/l, and 30-g/l sucrose and 6 g/l agar and pH were adjusted to 5.7 before autoclaving. The incubation conditions were at 25 ±2°C, photoperiod 16 hr. using Philips cool white fluorescent tubes of 1500 lux. Meristems–derived shoots were subcultured monthly into fresh medium to get aseptic plant mateials.

***In vitro* Storage:** To asses the effect of osmotic stress medium on mid–term storage of *in vitro* grown strawberry culture, the proliferated shoots were transferred to Knop's medium with and without hormones and supplemented with different concentration (0.1, 0.2 and 0.4 M) of mannitol and sorbitol and the cultures were incubated at 4°C under dark condition. Each concentration composed of four replications; each consisted of 10 explants. Survival (%), number of shoots and number of roots per explants were registered at 4, 6, 10, 14 and 15 months of storage. Also, percentage of growth recovery was calculated after 15 months of storage. In this respect, individual explants were transferred to fresh Knop's medium containing hormones and incubated at 25 ±2°C and survival % was assessed. The experiment was designed in complete randomized design and obtained data were statistically analyzed according to Waller and Duncan[31].

RAPD Analysis: Several preliminary experiments were performed to optimize the RAPD protocol. Some of these experiments are described in the results section of this paper. Based on the results of these preliminary experiments, a standard protocol was developed and used for subsequent experiments. DNA extraction was carried out using leaf materials collected from each variety. Genomic DNA was extracted and purified using the DNeasy plant Mini Kit following the manual instructions (QIAGEN, Chatsworth, CA). Five–mer oligonucleotide primers (Operon technology, USA) were used (Table 1). PCR reactions were performed in a 0.5 ml microcentrifuge tube containing 5 l of rat DNA (5 ng/l), 1 l of the ten base primer (15 ng/l), 0.5 l of Taq DNA polymerase (AmpliTaq, Perkin Elmer Cetus 5U/l), 11.5 l water and 7 l of a 3.57X buffer solution. The 3.57X buffer solution is freshly made by adding 280 l of a solution of ATP, TTP, CTP, and GTP (2.5 mM each) and 280 l of MgCl (10 mM) to 350 l of the 10X buffer supplied with the Taq polymerase. The reaction mixture was vortexed and centrifuged briefly and 50 l of mineral oil was overlaid on top of the aqueous layer.

Table 1: Sequence of amplified products of five abitrary primers (Operon Technologies) to generate RAPD markers in strawberry.

Primer	Sequence	Number of bands
OPA-11	5`CAATCGCCGT 3`	11
OPA-19	5`CAAACGTCGG 3`	15
OPB-06	5` TGCTCTGCCC3`	10
OPC-01	5` TTCGAGCCAG 3`	10
OPC-09	5` CTCACCGTCC 3`	8

PCR was initiated by denaturation step at 94C for 1 min and then the reaction was subjected at 44 cycles of 94C for 30 sec., 36C for 1 minute, and 72C for 2 minutes. A final elongation step of 2 minutes at 72C were performed. In order to select the optimal conditions of the RAPD-PCR different optimization experiments were carried out. Samples were stored at 4C after the final step. The amplification products were resolved by electrophoresis on a 1.5% agarose gel with ethidium bromide and visualized under UV. One marker was used 100 bp gives band from 100 bp to 12.000 Kb Photography was made by using Polaroid film type 57 (ASA3000)

RESULTS AND DISCUSSION

Storage at 4°c and Osmotic Stress Medium: Data presented in Table (2) show that up to 76 % of shoot culture remain healthy and green after 15 months storage on hormone-free medium supplemtented with 0.2 M sorbitol, while the lowest survival rate 20.43 % was observed on medium ammended with 0.1 M sorbitol and 1.0 mg/l BAP**.** However, up to 65 % of shootlet remain healthy and green on medium with 0.2 M mannitol without hormones at same period of storage (15 months). While the survival rate was 20.67 % on medium contained 0.2 M mannitol and 1.0 mg/l BAP (Table 2 and Fig. 1).

In general using sorbitol as osmotic stress seems to be more suitable for slow growth preservation of

Table 2: Percentage of survival leaflets per explant of Camarosa *Fragaria ananassa* variety during 4°C and dark storage at different concentrations of sorbitol and manitol.

Period	Sorbitol					Mannitol				
Treatment	Months					Months				
	4	6	10	14	15	4	6	10	14	15
0.1 M	100a	94.3c	52.0p	49.5q	55.8o	100a	95.4 b	55..9o	55..9o	52.8p
0.2 M	100a	100 a	74.0h	38.2 t	76.2fg	100a	100 a	70.3 J	67.0 k	65.3 l
0.4 M	100a	76.6 f	47.3r	47.3 r	47.3 r	100a	100 a	56.0 o	56.0 o	38.8 t
0.1M+IBA	100a	69.5 j	71.7 I	25.8x	20.4 z	100a	62..3m	65.7 l	46.0 s	33.9u
0.2M+IBA	100a	85.0d	81.3e	22.1y	21.3 yz	100a	60.8n	60.8 n	30.0 v	20.6z
0.4 +IBA	100a	100 a	75.3g	50.0q	27.8 w	100a	100 a	71.6 I	27.8w	20.8z

*Means having the same letter(s) are not significantly different (p<o.o5).

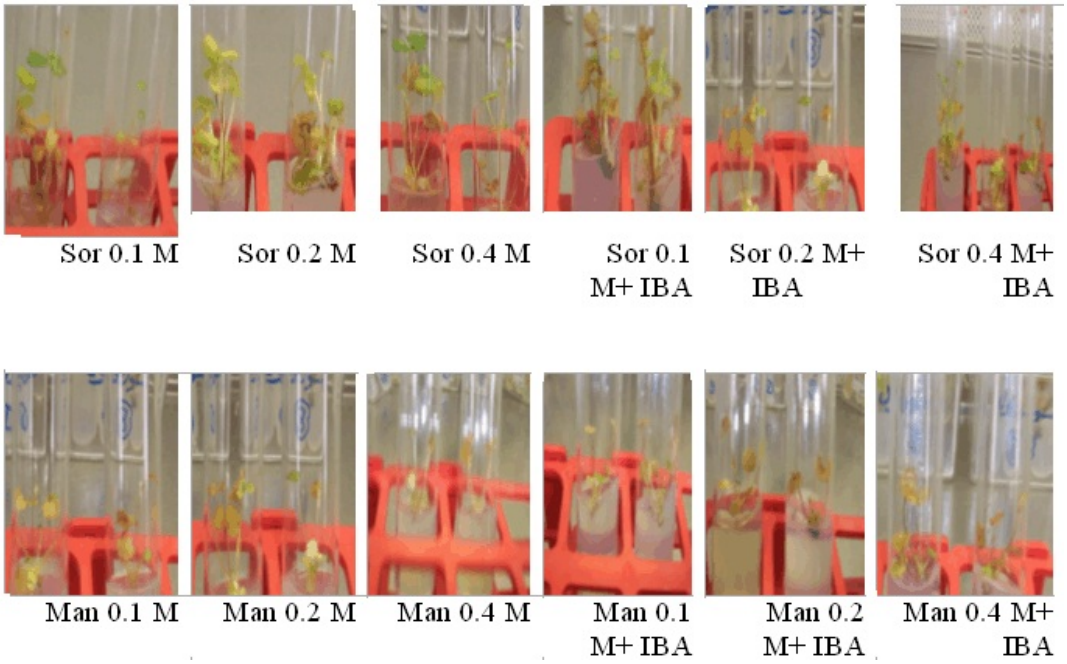

Fig. 1: Survived plantlets of Chandler (Fragaria ananassa) variety after 15 months of 4°C and dark storage at different concentrations of sorbitol and manitol.

strawberry tissue cultures since it showed high survival rate compared with manitol. In this respect, Flecher[9] mentioned mentioned that the cultures of asparagus remained viable after 15-16 months of storage on a medium consisting of MS media with the addition of 3% sucrose and 4% sorbitol and incubated at 6°C . Hae Boong *et al*.[13] found that the best conditions for *in vitro* storage of strawberry germplasm were at 2°C in light. However, Reed[27] mentioned that cold storage is important for managing *in vitro* germplasm collection of strawberry. Shoot cultures can typically be held at 4°C for 9 to 24 months before they require repropagation. Moreover, explants of 22 strawberry varieties were preserved at 4°C for 4 months with a survival percentage of over 50 % by Yu GuiHong *et al*. They mentioned that using combinations of osmotic regulators or growth inhibitors and low temperature storage may be of benefit.

On the other hand, addition of sorbitol to culture medium showed an increase in shoot number compared with mannitol. Strawberry plantlets performance an increment in producing shoots after 15 months on medium containing 0.1 M sorbitol supplemented with hormone followed by 0.2 M sorbitol without hormones and 0.2 M sorbitol supplemented with hormone. This result are in agreement with those of Golmirzaie and Toledo[11] which demonstrated that the use of sorbitol as an osmotic agent can be metabolized

Table 3: Number of new shoots per explant of Camarosa *Fragaria ananassa* variety during 4 °C and dark storage at different concentrations of sorbitol and manitol.

Period	Sorbitol					Manitol				
Treatment	Months					Months				
	4	6	10	14	15	4	6	10	14	15
0.1 M	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E	1 . 0 0 E
0.2 M	1.00 E	1.00 E	1.00 E	1.20 C	2.0 B	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E
0.4 M	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E
0.1M + IBA	1.00 E	1.00 E	1.00 E	3.00 A	3.00 A	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E
0.2M + IBA	1.00 E	1.00 E	1.00 E	1.167 D	1.43 D	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E
0.4M + IBA	1.00 E	1.00 E	1.33 D	1.33 D	1.33 D	1.00 E	1.00 E	1.00 E	1.00 E	1.00 E

*Means having the same letter(s) are not significantly different (p<o.o5)

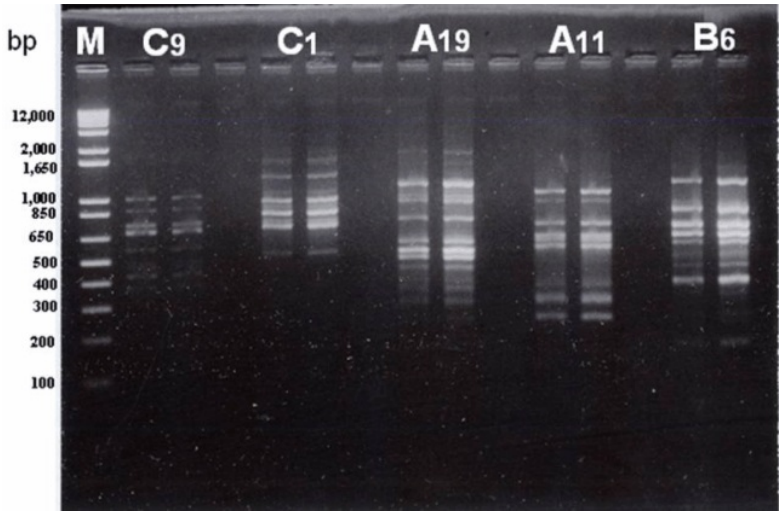

Fig. 2: Gel electrophoresis of RAPD fragments detected with the primer A11, A19, B6, C1 and C9 for Camarosa variety (preserved and non preserved explants). (M) Molecular marker (1Kb, 100-12,000Kb).

by the plantlets after few months of storage and exhibition an incremental growth rate, effectively reducing storage time (Table 3). In the same line, Espinosa *et al.*[8] stated that addition of mannitol reduced the growth of Ipomoea batatas plants. Also, Jarret and Gawel[14] mentioned that the addition of sorbitol and mannitol to culture media generally produced undesirable effects on gross plant morphology and loss of apical dominance.

Concerning roots number, the obtained results are viewing high value with sorbitol than mannitol. Percentage increase of root was noticed with medium containing 0.1M sorbitol followed with 0.1M mannitol and 0.2 M sorbitol (Table 4). Strawberry plantlets resume normal growth when placed in a culture medium without sorbitol as osmotic stress, in contrary manitol showing a decrease in plantlet recovery when placed on osmotic-free medium (Table 5).

RAPD Analysis for Genetic Stability: Variation among culture types for optimal storage conditions adds to the complexity of storing *in vitro Fragaria* germplasm collections. The effect of growth regulators, photoperiod and cold acclimatization on genetic stability in cold and osmotic stress storage were examined. Assessment of stability by RAPD was performed, with DNA extracted from five different *in vitro* explants preserved in 4°C and in dark conditions.

Table 4: Root number of Camarosa *Fragaria ananassa* variety during 4°C and dark storage at different concentrations of sorbitol and manitol.

Period	Sorbitol					Mannitol				
Treatment	Months					Months				
	4	6	10	14	15	4	6	10	14	15
0.1 M	0.0 E	0.0 E	2.86 A	2.93 A	2.93 A	0.0 E	0.0 E	1.96 C	2.43 B	2.43 B
0.2 M	0.0 E	0.0 E	1.76 D	1.86 CD	1.86 CD	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E
0.4 M	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E
0.1M +IBA	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E
0.2M +IBA	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E
0.4M +IBA	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E	0.0 E

*Means having the same letter(s) are not significantly different (p<o.o5)

Amplification patterns of preserved material were compared with the non preserved *in vitro* explants. Sequence of amplified products of five arbitrary primers is shown in Table (1). The reproducibility of RAPD appears to be highly influenced by the experimental conditions. It is therefore essential to optimize the PCR conditions to obtain reproducible results before going on routine analysis. Investigating each factor individually, such as genomic DNA quality and concentration, primer annealing and extension temperature is a perquisite. Consequently, series of preliminary experiments were conducted to select the suitable primers (Table 1) and the optimal conditions for RAPD analysis. One DNA markers were used, 1Kb (100bp-12000Kb) from New England Biolabs.

Primer (B6) amplified a total number of 10 bands in strawberry (Camarosa variety) preserved and non-preserved explants, the smallest size of the amplified products was 116.7 bp in all accessions (Fig. 2). The largest size of the amplified products was 1108.3 bp in all treatments. The analysis of this RAPD marker did not show any variation among the preserved and non preserved material. Primer (A11) amplified a total number of 11 bands in all material of strawberry (Camarosa variety) preserved and non-preserved explants. The largest size of the amplified products was 1000 bp in all treatments. The smallest size of the amplified products was 225 bp in all accessions (Fig. 2). The analysis of the stored and non-stored cultures was typical. Primer (A19) amplified a total number of 15 bands in strawberry (Camarosa variety) preserved and non-preserved explants. The largest size of the amplified products was 1825 bp and the smallest size of the amplified products was 300 bp in in all treatments (Fig. 2). The comparison of DNA patters of

Table 5: Recovery of Camarosa *Fragaria ananassa* shootlet after 15 month's storage at different concentrations of osmotic stress induced by sorbitol and manitol.

	Recovery (%)	
Treatment	Sorbitol	Mannitol
0.1 M	100 A	33.3 D
0.2 M	100 A	50 B
0.4 M	100 A	40 C
0.1M + IBA	100 A	100 A
0.2 M + IBA	100 A	00.0 E
0.4 M + IBA	100 A	33.3 D

*Means having the same letter(s) are not significantly different (p<o.o5)

preserved and non preserved material did not allow detecting any polymorphism caused by the preservation duration. Primer (C1) amplified a total number of 10 bands in strawberry (Camarosa variety) preserved and non-preserved explants. The largest size of the amplified products was 2000 bp in all treatments. The smallest size of the amplified products was 537.5 bp in all accessions (Fig. 2). The results showed no significant differences between preserved and non preserved material. Primer (C9) amplified a total number of 8 bands in strawberry (Camarosa variety) preserved and non-preserved explants. The largest size of the amplified products was 1000 bp in all treatments .The smallest size of the amplified products was 612 bp in all accessions (Fig. 2). The bands appeared to be similar in all treatment for both preserved and non preserved explants.

The present results are similar to that reported by Bekheet[1] in his study on *in vitro* conservation of Globe artichoke. According to RAPD analysis, plantlets derived from the *in vitro* preserved shoot buds were genetically identical to the control (non-preserved

buds). In this connectiont, genetic marker analysis has been used to study the degree of genetic change in plants regenerated *in vitro* such as pea[3], sugarbeet[28] and wheat[2].

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