MID-TERM STORAGE AND GENETIC STABILITY OF STRAWBERRY TISSUE CULTURES

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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 (Reed, 1991). This technique can be used for the multiplication and storage of disease-free plant germplasm. *In vitro* conservation is used as a complementary method to field maintenance and involves the sustainment of explants in a pathogen-free environment and the distribution of clonal material. It is widely used for the conservation of species which produce recalcitrant or no seeds, and for vegetatively propagated plant material (Engelmann, 1997). 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 (Withers, 1984). Among the different methods of *in vitro* preservation is the short- and medium-term conservation. Short-term conservation requires the transfer of *in vitro* material to fresh media every 2-3 months. Whereas, with medium-term conservation there is a need to lengthen the period between subcultures by reducing growth rate. This might be achieved by the use of modified environmental conditions (Withers, 1991), modified culture medium (Withers, 1987), growth retardants (Gupta, 2001; Mandal, 1995), osmotic regulators (Westcott, 1981; Espinoza *et al.,* 1984, Ng and Hahan, 1985; Monette, 1986**;** Mandal, 1999; Zamora and Paet, 1999) and/or reduction of oxygen concentration (Dekker *et al.,* 1991).

Slow growth conservation *via in vitro* cultures has been reported in many species (Withers and Engelmann 1998). Low temperature (2-10°C) for minimal growth storage of cultured plant cells and organs has been applied successfully to grape (Morel, 1975) and apple (Lundergan and Janick, 1979). The addition of osmoticums or growth retardants to the medium has proved efficient for reducing growth rates of different plants species. Osmoticums such as mannitol or sorbitol reduce mineral uptake by cells through differences in osmotic pressures thereby retarding plant growth (Dodds and Roberts, 1985; Thomson *et al.,* 1986).

The application of DNA technology in agricultural research has progressed rapidly over the last twenty years, especially in the area of variety identification. Polymerase Chain reaction (PCR) based techniques have become increasingly popular for fingerprinting and variety identification (Mulis and Faloona, 1987; Saiki *et al*., 1985). Random Amplified Polymorphic DNA (RAPD) markers, utilizing PCR amplification from single arbitrary primers, were developed by Williams and his co-workers (Williams *et al*., 1990). Dominant RAPD-markers have been used for the identification of different plant species, as well as for assessing genetic diversity (Graham *et al*., 1994; Moreno *et al*., 1995).

Vegetatively propagated strawberry plants are stored as *in vitro* plantlets for plant breeding, virus-free planting stock, and for germplasm preservation (Mullin and Schegel, 1976; Reed and Hummer, 1995). The most widely applied technique for medium-term storage is through temperature reduction, combined with a reduction in the concentration of nutritive elements, decrease in light intensity or storage in the dark. In this respect, high survival rate of strawberry plantlets was obtained from successful storing at 4°C in the dark for 12 and 24 months (Reed, 1991&1992). The present study aims to develop a procedure for *in vitro* mid-term storage of strawberry by investigating the effect of addition of sorbitol and mannitol to the culture medium and incubation at low temperature in addition to determining the genetic stability of stored cultures using RAPD analysis.

MATERIALS AND METHODS

Plant material and tissue culture

Strawberry (*Fragaria x ananassa*) variety Camarosa was used in this study. 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 distilled water. Tissue cultures were initiated from shoot tips consisting of meristem plus 2 to 3 leaf primordials which were excised and cultured onto glass tubes (100 x25 mm) containing Knop's medium supplemented with 1.0 mg/L Indolbutyric acid (IBA) and 30 g/L sucrose and 6 g/L agar and the pH was adjusted to 5.7 before autoclaving. The incubation conditions were at 25 ±2°C with photoperiod of 16 hr of light using Phillips cool white fluorescent tubes (1500 Lux). Meristem–derived shoots were subcultured monthly onto fresh medium to get aseptic plant materials.

In vitro storage

To asses the effect of osmotic stress medium on mid–term storage of *in vitro* grown strawberry cultures, the proliferated shoots were transferred to Knop's medium with and without hormones and supplemented with different concentrations (0.1, 0.2 and 0.4 M) of mannitol and sorbitol and the cultures were incubated at 4°C in the dark. Each concentration was composed of four replications; each replicate consisted of

10 explants. Survival percentage (%), number of shoots and number of roots per explant was registered after 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 the recovery media which consisted of fresh Knop's medium containing hormones and incubated at 25±2°C for nearly four weeks then recovery % was assessed. A complete randomized experimental design was used. The data were statistically analyzed according to (Waller and Duncan, 1969).

RAPD analysis

Several preliminary experiments were performed to optimize the RAPD protocol. 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 preserved and non preserved shootlets. Genomic DNA was extracted and purified using the DNeasy plant Mini Kit following the manual instructions (QIAGEN, Chatsworth, CA). Five ten- mer oligonucleotide primers (Operon technology, USA) were used. The DNA sequence of five arbitrary primers is given in (Table 1). PCR reactions were performed in a 0.5 ml microcentrifuge tube containing 5 µl of DNA (5 ng/µl), 1 µl of the ten base primer (15 ng/µl), 0.5 µl of Taq DNA polymerase (AmpliTaq, Perkin Elmer Cetus 5U/µl), 11.5 µl double distilled water and 7 µl of a 3.57X buffer solution. The 3.57X buffer solution was freshly made up by adding 280 µl of a solution of ATP, TTP, CTP, and GTP (2.5 mM each) and 280 µl of MgCl (10 mM) to 350 µl of the 10X buffer supplied with the Taq polymerase. The reaction mixture was vortexed and centrifuged briefly and 50 µl of mineral oil was overlaid on top of the aqueous layer.

PCR was initiated by a denaturation step at 94°C for 1 min and then the reaction was subjected to 44 cycles of 94°C for 30 sec., 36°C for 1 minute, and 72°C for 2 minutes. A final elongation step of 2 minutes at 72°C was performed. Samples were stored at 4°C after the final step. The amplification products were resolved by electrophoresis on a 1.5% agarose gel against a DNA size marker (Trackit ^{TM} 1 Kb plus DNA Ladder, Invitrogen) that is suitable for sizing DNA fragments ranging from 100bp-12 kb. The gel was stained with ethidium bromide and visualized on a UV transilluminator and photographed using a Polaroid camera (film type 57, ASA3000).

A series of preliminary experiments were conducted to select the suitable primers (Table 1) and the optimal conditions for RAPD analysis.

RESULTS AND DISCUSSION

Storage at 4°C on osmotic stress medium

Data presented in (Table 2) show that up to 76 % of shoot culture remained

healthy and green after 15 months storage on hormone-free medium supplemented with 0.2 M sorbitol, while the lowest survival rate (20.4%) was observed on medium amended with 0.1 M sorbitol and 1.0 mg/L IBA. However, up to 65 % of shootlets on medium with 0.2 M mannitol without hormones for the same storage period (15 months) survived but was yellowish in colour. While the lowest survival rate was (20.6%) on medium contained 0.2 M mannitol and 1.0 mg/L IBA (Table 2 and Fig. 1).

In general, using sorbitol as osmotic stress regulator seems to be more suitable for slow growth preservation of strawberry tissue cultures since it showed high survival rates of shootlets compared with mannitol. In this respect, Flecher (1994) 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*. (1996) found that the best conditions for *in vitro* storage of strawberry germplasm were at 2°C in the light. However, Reed (2002) mentioned that cold storage is important for managing *in vitro* germplasm collections of strawberry, where shoot cultures can typically be preserved at 4°C for 9 to 24 months before they require repropagation. Moreover, explants of 22 strawberry varieties were conserved at 4°C for 4 months with a survival percentage of over 50 % by Yu GuiHong *et al*. (2003)**.** They mentioned that using combinations of osmotic regulators or growth inhibitors and low temperature may prolong the period of conservation.

On the other hand, addition of sorbitol to culture medium with or without hormone (1 mg/L IBA) showed an increase in shoot number compared to media supplemented with mannitol (Table 3). The average number of shoots produced after 15 months on medium containing sorbitol varied from 1.33-3.00. Results show that the number of shoots increased after 10 months of storage on conservation media supplemented with sorbitol with or without hormone. Hence, it is observed that there is an increase in growth rate which ultimately will cause a reduction in storage period. While the addition of mannitol with or without hormone did not promote the formation of new shoots. These results are in agreement with those of Golmirzaie and Toledo (1997) which demonstrated that the use of sorbitol as an osmotic agent can be metabolized by the plantlets after few months of storage and exhibit an incremental growth rate, effectively reducing storage time. In the same context, Espinosa *et al.* (2002) stated that the addition of mannitol to the culture media reduced the growth of sweet potato (*Ipomoea batatas*) plants. Also, Jarret and Gawel, (2004) mentioned that the addition of sorbitol and mannitol to culture media generally produced undesirable effects on gross plant morphology and loss of apical dominance.

Root formation was observed on shootlets after six months of storage only

on media supplemented with sorbitol at the concentration of 0.1 M and 0.2 M and mannitol at 0.1 M (Table 4).The number of roots produced ranged from 1.86 to 2.93 by the end of 15 months conservation period. Shootlets in all other treatments lacked roots. Induction of both root and shoots are undesirable for medium-term conservation since it reduces the storage period before the need to transfer the material to fresh media.

Strawberry plantlets resumed a normal growth when placed in a culture medium without sorbitol as osmotic stress, contrarily, shootlets cultured on mannitol containing medium showed a decrease in plantlet recovery when placed on osmotic-free medium (Table 5).

It could be concluded from this study that the addition of sorbitol, as an osmotic stress regulator, to the culture media for medium term conservation gave better results than mannitol as exhibited by data on survival percentage of shootlets and percentage of recovery, despite the initiation of growth activity as observed by the increase in number of shoots and roots after 15 months of storage. While, the addition of mannitol gave weak and yellow shootlets after 15 months of storage which lead to reduction of the recovery percentage, in addition to the low survival rate during preservation period.

RAPD analysis for genetic stability

The effects of growth regulators, photoperiod and cold acclimatization on genetic stability under osmotic stress storage conditions were examined. Assessment of genetic stability was performed by RAPD analysis, with DNA extracted from five different *in vitro* explants preserved at 4°C in the dark. Amplification patterns of preserved material were compared with the non preserved *in vitro* explants.

RAPD profiles generated by the 5 arbitrary 10 mer primers (Table 1) are shown in Fig (2). Primer (B6) amplified a total number of 10 bands from DNA extracted from preserved and non preserved shootlets of strawberry var. Camarosa, the smallest size of the amplified products was 116.7 bp in all samples and the largest size was 1108.3 bp. Analysis of these RAPD reactions did not show any variation among the preserved and non preserved material. Primer (A11) amplified a total number of 11 bands in all DNA samples of strawberry, preserved and non preserved shootlets. The largest size of the amplified products was 1000 bp and the smallest size was 225 bp. The resultant RAPD profiles of the stored and non-stored cultures were typical. Primer (A19) amplified a total number of 15 bands. The largest size of the amplified products was 1825 bp and the smallest size was 300 bp. The comparison between DNA patterns of preserved and non preserved material showed no polymorphism due to the duration period of preservation. Primer (C1) amplified a total number of 10 bands in DNA samples of preserved and non preserved strawberry shootlets. The

largest size of the amplified products was 2000 bp and the smallest size of was 537.5 bp. The results showed no significant differences between preserved and non preserved materials. Primer (C9) amplified a total number of 8 bands in DNA of preserved and non preserved shootlets. The largest size of the amplified products was 1000 bp and the smallest size was 612 bp. The banding patterns appeared to be similar for both preserved and non preserved shootlets.

The present results are similar to that reported by Bekheet (2007) 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 context, molecular marker analysis has been used to study the degree of genetic change in plants regenerated *in vitro* such as pea (Cecchini *et al*., 1992), sugarbeet (Sabir *et al*., 1992) and wheat (Brown *et al*., 1993).

SUMMARY

The objectives of the present investigation were to establish an efficient medium-term *in vitro* preservation protocol for strawberry and to assess the genetic stability of the stored cultures. The medium term preservation study was initiated under 4°C and dark conditions using *in vitro* grown shootlet cultures. Shoot explants were subjected to different concentrations of mannitol and sorbitol (0.1, 0.2 and 0.4 M) as osmotic regulators supplemented to the culture medium which contained or was devoid of Indolbutyric acid (IBA). Results showed that the number of shoots increased when the culture medium was supplemented with 1 mg IBA/L and 0.1 M sorbitol and preserved for 15 months. However, the highest percentage of healthy shoots (76%) was observed with the medium containing 0.2 M sorbitol without IBA. Whereas, the highest number of roots per shoot occurred on the medium supplemented with 0.1 M sorbitol devoid of IBA. Also, sorbitol containing medium registered best results of shootlet recovery. Determination of stability was performed by assessment of randomly amplified polymorphic DNA (RAPD). The total number of amplified RAPD fragments varied depending on the sequence of the primers used. The generated RAPD profiles show no variation among the preserved and non-preserved material with all primers tested.

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Table (1): Sequence of the five 10-mer arbitrary primers (Operon Technologies) used in the RAPD reactions and the number of generated DNA fragments.

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

Table (2): Percentage of survived shootlets of *Fragaria ananassa* var. Camarosa conserved *in vitro* on culture medium containing different concentrations of sorbitol and mannitol at 4°C in the dark.

Period / Treatment	Sorbitol					Mannitol				
	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	100.0a	74.0h	38.2 t	76.2fg	100a	100.0a	70.3 J	67.0k	65.3l
0.4 M	100a	76.6 f	47.3r	47.3 r	47.3r	100a	100.0a	56.0 o	56.0o	38.8t
0.1 M+IBA	100a	69.5 j	71.7I	25.8x	20.4z	100a	62.3m	65.7 l	46.0 s	33.9u
0.2 M+IBA	100a	85.0d	81.3e	22.1y	21.3yz	100a	60.8n	60.8 n	30.0v	20.6z
0.4 M+IBA	100a	100.0a	75.3g	50.0q	27.8w	100a	100.0a	71.6 I	27.8w	20.8z

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

Table (3): Number of new shoots per explant of *Fragaria ananassa* var. Camarosa conserved *in vitro* on culture media supplemented with different concentrations of sorbitol and mannitol at 4°C in the dark.

Period / Treatment	Sorbitol					Mannitol				
	Months					Months				
	4	6	10	14	15	4	6	10	14	15
0.1 M	1.00e	1.00e	1.00e	1.00e	1.00e	1.00e	1.00e	1.00e	1.00e	1.00e
0.2 M	1.00e	1.00e	1.00e	1.20c	2.0 b	1.00e	1.00e	1.00e	1.00e	1.00e
0.4 M	1.00e	1.00e	1.00e	1.00e	1.00e	1.00e	1.00e	1.00e	1.00e	1.00e
0.1 M+IBA	1.00e	1.00e	1.00e	3.00a	3.00a	1.00e	1.00e	1.00e	1.00e	1.00e
0.2 M+IBA	1.00e	1.00e	1.00e	1.167d	1.43d	1.00e	1.00e	1.00e	1.00e	1.00e
0.4 M+IBA	1.00e	1.00e	1.33d	1.33 d	1.33d	1.00e	1.00e	1.00e	1.00e	1.00e

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

Table (4): Number of roots/ shootlets of *Fragaria ananassa* var. Camarosa cultured on media containing different concentrations of sorbitol and mannitol and stored at 4°C in the dark.

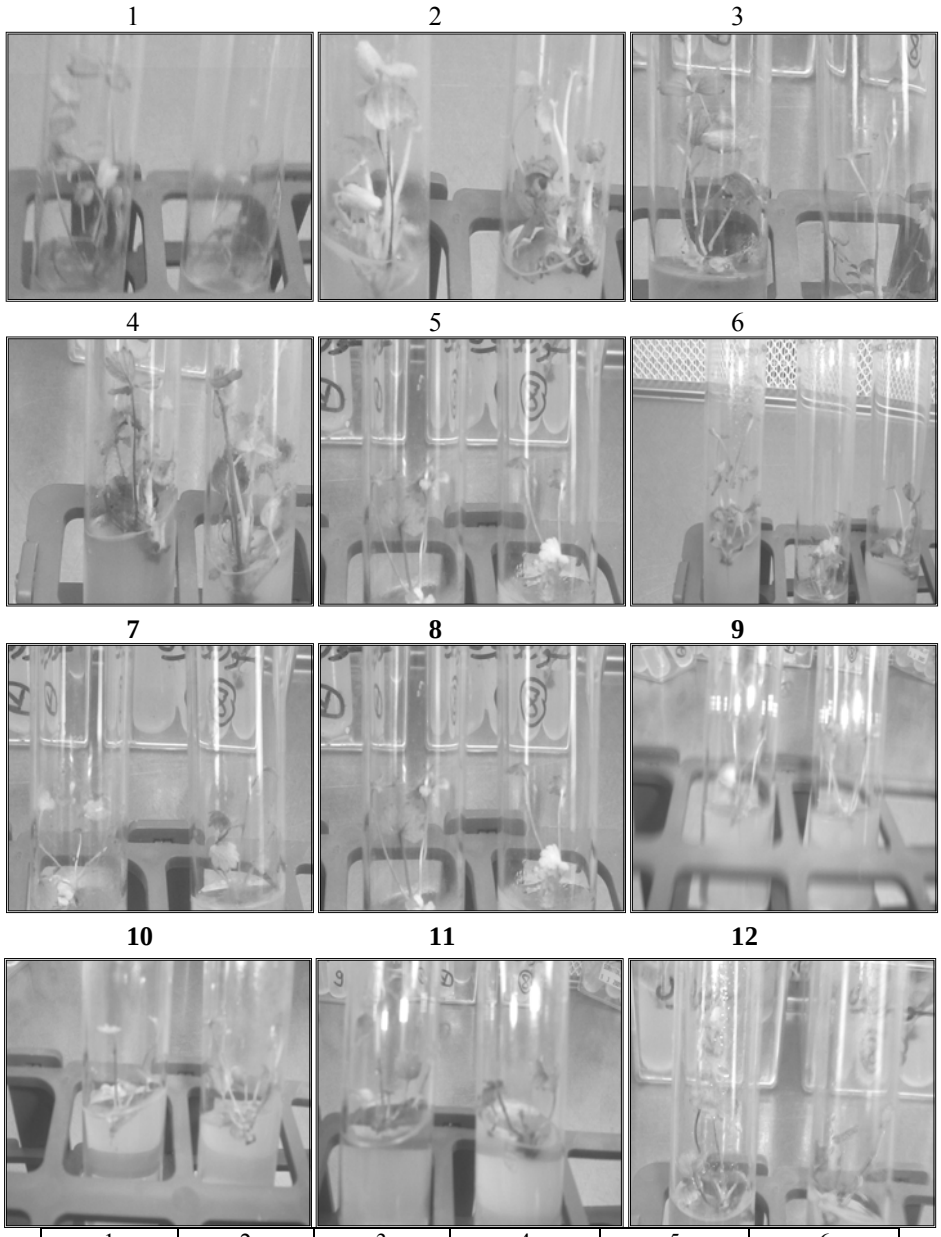
Period / Treatment	Sorbitol					Mannitol				
	Months					Months				
	4	6	10	14	15	4	6	10	14	15
0.1 M	0.0e	0.0e	2.86a	2.93a	2.93a	0.0e	0.0e	1.96c	2.43b	2.43b
0.2 M	0.0e	0.0e	1.76d	1.86cd	1.86cd	0.0e	0.0e	0.0e	0.0e	0.0e
0.4 M	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e
0.1 M+IBA	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e
0.2 M+IBA	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e
0.4 M+IBA	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e	0.0e

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

Table (5): Percentage of shootlets recovered from *Fragaria ananassa* var. Camarosa after 15 months storage under osmotic stress condition induced by sorbitol and mannitol.

Treatment	No. of survived shootlets/40 shootlets	Recovery % of sorbitol stressed shootlets	No. of survived shootlets/40 shootlets	Recovery % of mannitol stressed shootlets
0.1 M	22	100 a	21	33.3 d
0.2 M	30	100 a	26	50.0 b
0.4 M	19	100 a	15	40 .0c
0.1M + IBA	8	100 a	13	100 a
0.2 M + IBA	9	100 a	8	00.0 e
0.4 M + IBA	11	100 a	8	33.3 d

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



1	2	3	4	5	6
Sor 0.1 M	Sor 0.2 M	Sor 0.4 M	Sor 0.1 M+ IBA	Sor 0.2 M+ IBA	Sor 0.4 M+ IBA
7	8	9	10	11	12
Man 0.1 M	Man 0.2 M	Man 0.4 M	Man 0.1 M+ IBA	Man 0.2 M+ IBA	Man 0.4 M+ IBA

Fig. (1): Survived shootlets of *Fragaria ananassa* var. Camarosa after 15 months of conservation at 4°C in the dark on media containing different concentrations of sorbitol and mannitol.

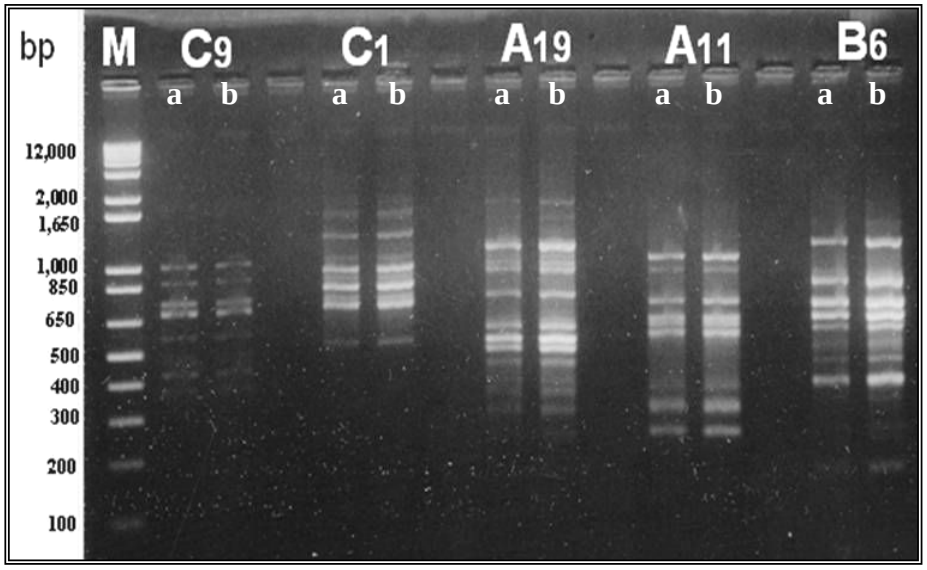


Fig. (2): Agarose gel (1.5%) showing RAPD profiles generated by the primers C9, C1, A19, A11 and B6 from DNA of (a) control (non preserved shootlets) (b) shootlets preserved on Knop's medium supplemented with M 0.2 sorbitol for 15 months .(M) Molecular marker (plus DNA Ladder 100-12,000 bp).