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Full Length Research Paper

Slow growth conservation and molecular characterization of *Deutzia scabra* Thunb.

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Possibility of long-term storage *in vitro* of *Deutzia scabra* Thunb. shoots and their genetic stability were evaluated. The effect of mannitol concentrations (20 and 40 g/L), presence and absence of activated charcoal (AC) and temperature (24 or 4°C) in MS medium were studied in respect to survival percentage, number, length, leaves number of axillary shoots and RAPD–based DNA fingerprints. The data revealed that the highest survival (%) was produced on medium without mannitol (irrespective of AC). The highest numbers of shoots were recorded on control medium and medium containing 20 g/L mannitol without AC after 3 months. The longest shootlets was recorded on medium without mannitol and with AC after 12 months. Shoots were recovery after different conservation periods; all treatments during the different conservation periods were live except shoots were incubated at 4°C after 12 months irrespective of AC and after 9 months without AC were dead. Preliminary RAPD analysis found no differences between plants stored for 12 months and the mother plants were conserved on medium without mannitol (irrespective of AC), while the differences preserved on mannitol treatments were found.

Key words: *In vitro* conservation, *Deutzia scabra* Thunb., mannitol, low temperature, activated charcoal and RAPD DNA.

INTRODUCTION

Deutzia scabra Thunb. is an ornamental shrub in the family Saxifragaceae. This shrub grows for showy, white to pink-tinged flowers appearing in spring or early summer. The deutzias thrive in almost any well-drained soil, and are well adapted for shrub borders. Potted plants forced with a temperature not exceeding 50°C develop into beautiful specimens for greenhouses (Baliy, 1976). *D. scabra* shrub plant in Egypt is considered very rare plant as it is located only at there gardens (Qubba Botanical, Qubba place; Orman Botanical and Zoo garden) (Khalifa and Loutify, 2006).

Propagation requires a cold greenhouse, seed germination taking 1 to 3 months at 18°C, when they are large enough to handle, prick the seedlings out into individual pots and grow them on in the greenhouse for at least their first winter. Plant them out into their permanent positions in late spring or early summer, after the last expected frosts (Sheat, 1948).

Clonally propagated plants are usually maintained in botanical gardens. Some of those plants are usually preserved by continuous multiplication of tubers, roots, cuttings or bulbs. Such a procedure is laborious and exposes plants to pests and environmental stresses. Tissue culture has proved to be a useful tool for storage of vegetatively propagated commercial crops like potato, palm, forest trees, fruit trees and other species (Dodds and Roberts, 1985; Jones et al., 1979).

Storage of shoot tips or meristem derived explants under slow rate of growth has a significant use in the international germplasm resources units. This procedure makes germplasm available at any times for international distribution (Wilkins and Dodds, 1983). Several types of plant materials have been used for *in vitro* preservation of clonally propagated crops.

The aim of this work was to evaluate the influence of temperature, mannitol concentrations and activated

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charcoal (AC) on duration of storage, post storage recovery with DNA analysis of resultant *in vitro* deutzias plantlets.

MATERIALS AND METHODS

This study has been carried out on *D. scabra* Thunb at the Tissue Culture and Germplasm Conservation Research Laboratory, Horticulture Research Institute, ARC Egypt, and Plant Biotechnology Department, National Research Center, Egypt.

Plant materials

The explants used in this study were taken from *D. scarba* Thunb. shrub grown on Orman Botanical Garden, Giza. Explants were soaked for 1 min in 70% ethanol solution under aseptic condition in laminar air-flow cabinet. After that they were treated with 10% (v/v) Clorox with a few drops of tween-20 for 15 min, followed by rinsing three times with sterile distilled water. After that they were immersed in 0.1% mercuric chloride (MC) with a few drops of tween-20 for 10 min. Finally they were rinsed three times with sterile distilled water. Explants were then cultured into growth regulator-free MS-medium (Murashige and Skoog, 1962). After three weeks free contamination explants were sub-cultured for one time (4 weeks) on multiplication MS-culture medium enriched with 2 mg/l benzyl adenine (BA), 25 g/L sucrose and 7.0 g/L agar as described by Sayed and Gabr (2009). The medium was adjusted to pH 5.7 ± 0.1, with 25 ml in 200 ml capacity glass jars before autoclaving at 121°C and 1.2 kg/cm^2 for 15 min shoots were aseptically sectioned into explants (microcutting at 1 to 1.5 cm length) for use in conservation treatments.

Culture conditions on shooting behaviour

Cultures were incubated at 24°C under 16-h photoperiod provided by fluorescent white light 50 µmol m^{-2} s^{-1} on control medium (MS medium supplemented with 2 mg/L BA and 25 g/L sucrose) with and without activated charcoal (AC) and mannitol (20 and 40 g/l) with or without AC. While in storage, the cultures were incubated in dark at 4°C with or without AC. All of these treatments were stored for different periods (3, 6, 9 and 12 month). Afterward, survival %, shoot number, shootlet length and leaves number were recorded.

Culture conditions after storage and subsequent shoot growth

After storage, shoots produced from treatments and periods were cultured on fresh MS medium enriched with 2 mg/L benzyl adenine (BA), 25 g/L sucrose and 7.0 g/L agar and incubated in growth chamber at 24°C under 16-h photoperiod provided by fluorescent white light 50 µmol m^{-2} s^{-1} for four weeks. Afterward, the survival %, shoots number, shootlet length and leaves number were calculated.

Experimental design and Statistical analysis

We used 40 replicates (40 jars) for each treatment and at the end of each conservation period we took 10 replicates (10 jars), calculated the survival %, new shoots number, shootlet length, leaves number/culture and tested the recovery after this storage period and subsequent shoot growth for the same 10 replicates after 4 weeks to calculate the survival %, new shoots number, shootlet length and leaves number/culture. Data were subjected to analysis of variance (ANOVA) and the means were separated where appropriate, using the least significant difference (LSD) at 5% significance.

Extraction and purification of genomic DNA

Leaves from *in vitro* survival plantlets were harvested after 12 months of preservation and DNA was isolated in order to perform RAPD analysis, to detect the variation a modified, CTAB (hexadecyl trimethyl ammonium bromide) procedure based on the protocol of Doyle and Doyle (1990).

RAPD analysis

A set of three random 15-mer primers was used in the detection of polymorphism among the 6 preservation treatments that survived after 12 months with the mother plant. These primers were synthesized on Metabion international AG (D-82152 Martinsried/ Deutschland). RAPD-PCR was carried out according to the procedure given by Williams et al. (1990) with minor modifications. The amplification reaction was carried out in 25 µl reaction volume containing 1X PCR buffer, 1.5 mM MgCl2, 2 mM dNTPs, 1 µM primer, 1 U *Taq* DNA polymerase and 25 ng templates DNA.

PCR amplification was performed in a Perkin-Elmer/GeneAmp® PCR System 9700 (*PE* Applied Biosystems) programmed to fulfil 40 cycles after an initial denaturation cycle for 5 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 1 min, annealing step at 36°C for 1 min, and an elongation step at 72°C for 1 min. The primer extension segment was extended to 7 min at 72°C in the final cycle.

The amplification products were resolved by electrophoresis in a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in 1X TBE buffer at 95 volts.

RESULTS AND DISCUSSION

In vitro conservation

Data on survival (%) of the conservation treatments and conservation periods were analyzed as shown in Table 2. The highest survival percentage (96.67%) was observed when the explants on control medium were cultured without AC at 24°C. While explants were incubated at low temperature (4°C) with and without AC, produced the lowest percentage of survival (41.67%). Concerning the effect of conservation periods recorded, the highest percentages of survival were observed after 3 months (99.17%), and increased periods of conservation from 6, 9 and 12 month decreased gradually the percentage survival (72.50, 58.33 and 52.50%, respectively). Our observations are in agreement with the earlier works, Witomska et al. (2008) reported that the better effects gave the storage of *Hosta* 'Blue Angel'. At 24°C after 12 months 60 to 65% shoots were in a good condition. At 6°C a number of viable shoots dropped after 12 months; 5 to 55% shoots survived, depending on a medium. Survival percentages were decreased with increased conservation periods from 2nd to 12th. Tyagi et al. (2009) reported that *in vitro* conservation by slow growth method was achieved on conservation medium (1/2 MS (major salts) +5 µM BAP +0.7% agar); about 70% of the cultures

Table 1. Primers used and their annealing temperatures.

Primer-	Sequence 5'--------- 3'
AM-7	CTTCGGCAGCATCTCTTCAT
AM-8	CAGTGTGGAAGCCGATTATG
AM-9	ATGTGTTGTCTGGCTTGGTA

Table 2. Effect of different treatments and periods on survival (%) of *in vitro D. scabra* under conservation.

Conservation treatments	Conservation periods (months)				Mean (B)
	3	6	9	12	
Control	100.00	93.33	100.00	93.33	96.67
Control + AC	100.00	80.00	73.33	73.33	81.67
Mannitol 20g/l	93.33	86.67	86.67	80.00	86.67
Mannitol 20g/l +AC	100.00	86.67	80.00	60.00	81.67
Mannitol 40g/l	100.00	73.33	26.67	20.00	55.00
Mannitol 40g/l +AC	100.00	66.67	80.00	73.33	80.00
Low temp. 4° C	100.00	40.00	13.33	13.33	41.67
Low temp. 4° C + AC	100.00	53.33	6.67	6.67	41.67
Mean (A)	99.17	72.50	58.33	52.50	

$LSD_{0.05}$ A = 10.66; B = 15.08; A x B = 30.15.

survived up to 18 months at 25 ± 2°C.

Shoots during storage

Data in Table 3 revealed that the highest values for shoots number/culture (2.53 and 2.44) were recorded when the explants were cultured on both control medium and medium with 20 g/L mannitol without AC respectively, at 24°C. While storage at low temperature (4°C) gave the negative effects on shoots number/culture irrespectively of AC. Concerning the period of conservation, after 3 months cultures gave the largest number of shoots (2.06), after 6 and 9 months shoots number were similar (1.70 shoots/culture), falling significantly after 12 months of storage (1.51 shoots/culture).

More shoots, which were longer and had larger number of leaves/culture, were obtained at 24°C as compared to 4°C, in the presence of AC as compared with the absence of it and without mannitol than with it. The longest shootlets and the highest number of leaves were observed when the explants stored in control media supplemented with AC and at 24°C. While, Cultures stored at low temperature (4°C) had shorter shoots (0.33 cm) and fewer leaves per culture. After 9 and 12 months of storage, shoots were more longer than leaves after 3 and 6 months.

Our observations are in agreement with Witomska et al. (2008) that storage temperature affected number, length and quality of axillary shoots of Hosta 'Blue Angel'. More shoots, which were longer and of better quality, was obtained at 24°C as compared to 6°C. Falling significantly was after 12 months of storage while shoot quality kept decreasing steadily between 2nd and 12th month. Gabr (2008) reported that shoots number was significantly increased by increasing the periods of preservation at all treatments used, except using low temperature (5°C) or when the media were contained with the highest concentrations (120 g/L) of mannitol or sucrose.

Thomas (2008) reported that the effects of AC on morphogenesis may be mainly due to its irreversible adsorption of inhibitory compounds in the culture medium and binding the toxic metabolites. The effect of AC on growth regulator uptake is still unclear but some workers believe that AC may gradually release certain adsorbed products, such as nutrients and growth regulators which become available to plants.

In vitro recovery

Survival %: It was possible to store *D. scabra* cultures for twelve months with a high survival percentage after recovery, regardless of the storage treatments. Data presented in Table 4 revealed that, the highest percentage of survival cultures were regenerated from conservation treatments (71.67, 71.67, 71.67, 68.33 and 70.00%, respectively) which were found in control, mannitol 20 g/L with and without AC and mannitol40 g/L only with AC. On the other hand, cultures were

Table 3. Effect of treatments and periods of conservation on shooting of *in vitro D. scabra* under storage conditions.

Conservation treatments	Storage periods (months)				Mean B
	3	6	9	12	
	Shoots number/explant[a]				
Control	2.92	3.08	2.84	1.27	2.527
Control + AC	1.53	2.58	1.92	1.20	1.808
Mannitol 20 g/L	2.43	1.62	1.96	1.47	1.868
Mannitol 20 g/L + AC	1.47	1.43	1.84	1.42	1.539
Mannitol 40 g/L	4.52	1.50	2.50	1.22	2.437
Mannitol 40 g/L + AC	1.60	1.35	1.83	1.63	1.604
Low temp.	1.00	1.00	0.33	0.67	0.750
Low temp. + AC	1.00	1.00	0.33	0.33	0.667
Mean A	2.059	1.696	1.695	1.510	
	Shootlet length (cm)[b]				
Control	2.07	3.66	4.37	4.57	3.667
Control + AC	3.88	3.64	4.63	4.67	4.205
Mannitol 20 g/L	1.17	1.21	3.87	3.90	2.537
Mannitol 20 g/L + AC	1.60	1.72	2.46	2.50	2.070
Mannitol 40 g/L	0.62	1.00	1.33	1.50	1.113
Mannitol 40 g/L + AC	1.23	1.50	2.06	2.10	1.722
Low temp.	0.50	0.50	0.17	0.17	0.333
Low temp. + AC	0.50	0.50	0.17	0.17	0.333
Mean A	1.445	1.717	2.382	2.446	
	Number of leaves /Shootlet[c]				
Control	6.56	7.33	8.47	8.50	7.71
Control + AC	8.94	7.64	9.63	9.33	8.89
Mannitol 20 g/L	4.72	6.13	9.56	9.33	7.44
Mannitol 20 g/L + AC	6.53	6.37	7.59	7.63	7.03
Mannitol 40 g/L	4.67	5.28	5.00	5.34	5.07
Mannitol 40 g/L + AC	6.04	4.44	8.02	7.67	6.54
Low temp.	2.00	3.56	0.67	0.67	1.72
Low temp. + AC	2.00	2.00	0.67	0.67	1.33
Mean A	5.182	5.344	6.200	6.142	

[a]LSD 0.05 A: 0.3672 B: 0.5193 A×B: 1.0390; [b]LSD 0.05 A: 0.2663 B: 0.3766 A×B: 2.4460; [c]LSD 0.05 A: 0.6610 B: 0.9348 A×B: 1.8700.

incubated at low temperature (4°C), which gave the lowest percentage of cultures survival (30.00%) and were incubated for 9 and 12 month without AC and for 9 month with AC, completely field to recovery (died). For the effect of different periods of conservation survival percentage of regeneration cultures, the highest survival percentage (94.17%) was found after 3 months. The survival percentage of regeneration culture gradually decreased as the periods of conservation increased from 6 to 12 month which ranged from (55.83 to 35.00%).

Shooting behaviors after recovery

The data in Table 5 show that no significant differences between treatments conserved at 24°C with high regenerated shoots compared with cultures stored at low temperature (4°C) irrespective of AC, which gave

Table 4. Effect of conservation treatments and periods on survival percentage of *in vitro D. scabra*, after recovery.

Conservation treatments	Conservation periods (months)				Mean (B)
	3	6	9	12	
Control	100.00	73.33	66.67	46.67	71.67
Control + AC	100.00	66.67	66.67	53.33	71.67
Mannitol 20 g/L	80.00	66.67	66.67	60.00	68.33
Mannitol 20 g/L +AC	93.33	73.33	66.67	53.33	71.67
Mannitol 40 g/L	100.00	13.33	20.00	13.33	36.67
Mannitol 40 g/L +AC	100.00	6.00	66.67	53.33	70.00
Low temp. 4° C	93.33	26.67	0.00	0.00	30.00
Low temp. 4° C + AC	86.67	66.67	6.67	0.00	40.00
Mean (A)	94.17	55.83	45.00	35.00	

$LSD_{0.05}$ A: 9.975; B: 14.11; A x B: 28.21.

Table 5. Effect of treatments and periods of conservation on shooting behaviors of *in vitro D. scabra*, after recovery.

Conservation treatments	Conservation periods (months)				Mean B
	3	6	9	12	
	Shootlet number/explant[a]				
Control	1.47	1.31	1.27	1.23	1.32
Control + AC	1.80	1.22	1.20	1.13	1.34
Mannitol 20 g/L	2.10	1.29	1.47	1.40	1.56
Mannitol 20 g/L + AC	1.70	1.07	1.28	1.23	1.32
Mannitol 40 g/L	2.80	1.00	0.67	0.67	1.28
Mannitol 40 g/L + AC	2.00	0.75	1.63	1.57	1.49
Low temp.	1.77	0.77	0.00	0.00	0.64
Low temp. + AC	1.10	1.76	0.33	0.00	0.80
Mean A	1.84	1.15	0.98	0.90	
	Shootlet length (cm)[b]				
Control	0.84	1.25	1.87	1.83	1.45
Control + AC	1.27	1.64	1.77	1.73	1.60
Mannitol 20g/l	1.04	1.93	1.77	1.70	1.61
Mannitol 20g/l + AC	0.98	1.89	1.87	1.80	1.64
Mannitol 40g/l	1.32	1.17	1.00	1.00	1.12
Mannitol 40g/l + AC	1.13	1.39	1.90	1.27	1.42
Low temp.	0.91	1.06	0.00	0.00	0.49
Low temp. + AC	0.97	1.77	0.53	0.00	0.82
Mean A	1.06	1.51	1.34	1.17	
	Number of leaves/Shootlet[c]				
Control	5.40	6.22	6.17	6.10	5.97
Control + AC	4.80	4.61	4.57	4.50	4.62
Mannitol 20 g/L	4.71	5.78	5.07	5.67	5.31
Mannitol 20 g/L + AC	5.13	5.56	5.53	5.50	5.43
Mannitol 40 g/L	5.47	3.33	3.00	2.67	3.62
Mannitol 40 g/L + AC	4.93	4.10	5.63	5.57	5.06
Low temp.	4.67	2.89	0.00	0.00	1.89

Table 5. Contd.

Low temp. + AC	4.67	5.06	1.33	0.00	2.76
Mean A	4.97	4.69	3.91	3.75	

[a]LSD 0.05 A: 0.227 B: 0.321 A×B: 0.643; [b]LSD 0.05 A: 0.299 B: 0.423 A×B: 0.847; [c]LSD 0.05 A: 862 B: 1.219 A×B: 2.439.

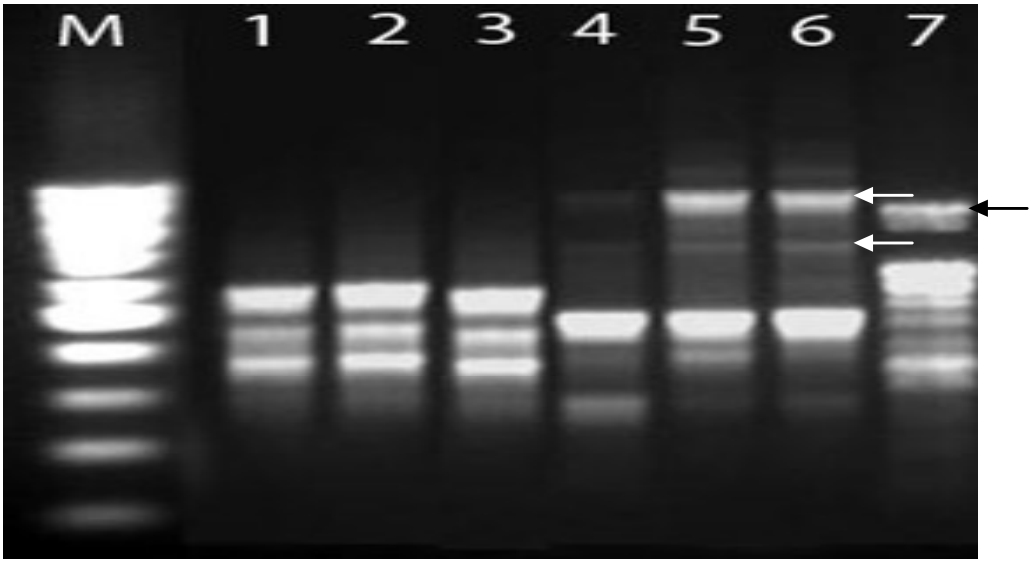

Figure 1. RAPD pattern of the *in vitro* preservation treatments of *Deutzia scabra* with primer 1. M. DNA marker (100 - 1500 pb). (1) Mother plant. (2) Control. (3) Control + AC (4) 20 g/l mannitol. (5) 20 g/l mannitol + AC (6) 40 g/l mannitol. (7) 40 g/l mannitol + AC.

negative significance on shoots regeneration after recovery. Regarding the shoots regenerated from different conservation periods, the number of shoots regenerated gradually decreased by increasing the periods of conservation from 3 to 12 month which ranged from 1.84 to 0.90 shoots/culture.

It is quite clear from Table 5 that the longest shootlets length were regenerated from conservation treatments (1.60, 1.61 and 1.63 cm), which were observed when regenerated from control medium with AC and medium with 20 g/L mannitol with and without AC. The largest leaves number regenerated from different treatments of conservation (6.0 leaves/culture) were observed on control medium without AC. On the other hand, the shoots regenerated from cultures incubated under low temperature (4°C) gave the shortest regeneration shootlets (0.49 cm) and the lowest number of leaves (1.9 leaves/culture).

In the same way, previous studies were in agreement with our results, Tyagi et al. (2009) recorded that successful re-growth of plants on micropropagation medium was obtained by culturing nodal explants excised from 18-months old conserved plants. Some 96% of the plants survived the hardening treatment and grew normally in a greenhouse. Gabr (2008) reported that the highest survival percentage on recovery of explants was recorded when shoots were preserved under normal conditions, 3 g/L activated charcoal. Witomska et al. (2008) studied the influence of storage conditions on number and length of the shoots formed during the following 3 culture passages. The highest number of axillary shoots was obtained during the first passage after storage, irrespectively of storage length and conditions. On the other hand, Bekheet (2000) reported that in asparagus (*Asparagus officinalis*) storage at low temperature was successful at 5°C, in darkness, 100% plantlets fully recovered after 6 months, in 90% after 12 months and in 50% after 18 months.

RAPD analysis of the regenerated plants after conservation

Three random primers (AM1, AM2 and AM3) were screened in RAPD analysis for their ability to produce sufficient amplification products. The results of DNA fingerprints generated by PCR amplification using the three primers are presented in Figures 1, 2 and 3. RAPD profiles of the conserved cultures were preserved for 12 months on control medium with or without AC and the

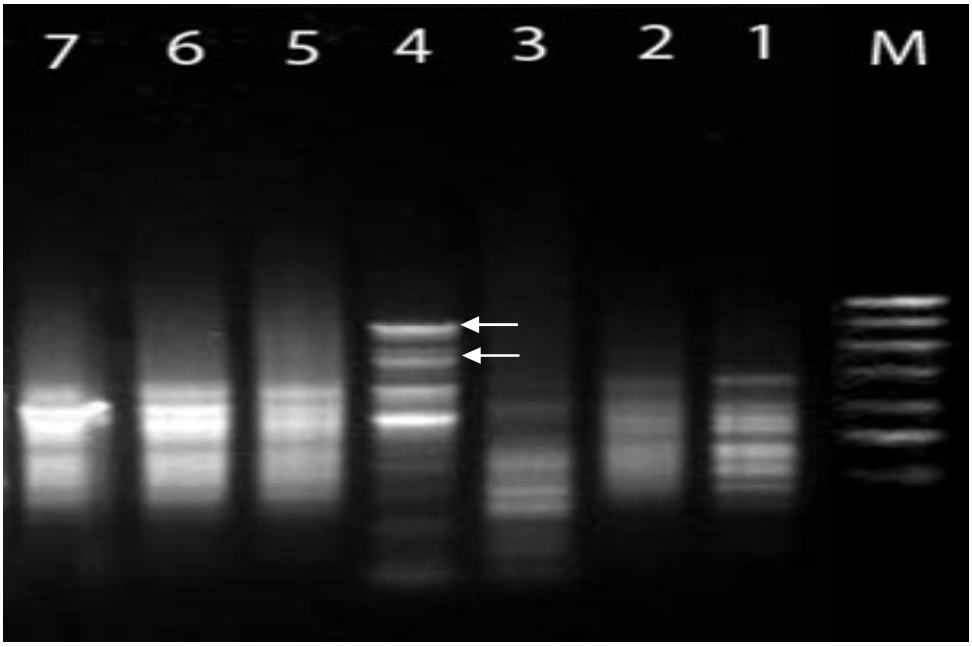

Figure 2. RAPD pattern of the *in vitro* preservation treatments of *D. scabra* with primer 2. M. DNA marker (100 - 1500 pb). (1) Mother plant; (2) Control; (3) Control + AC; (4) 20 g/L mannitol; (5) 20 g/L mannitol + AC; (6) 40 g/L mannitol; (7) 40 g/L mannitol + AC.

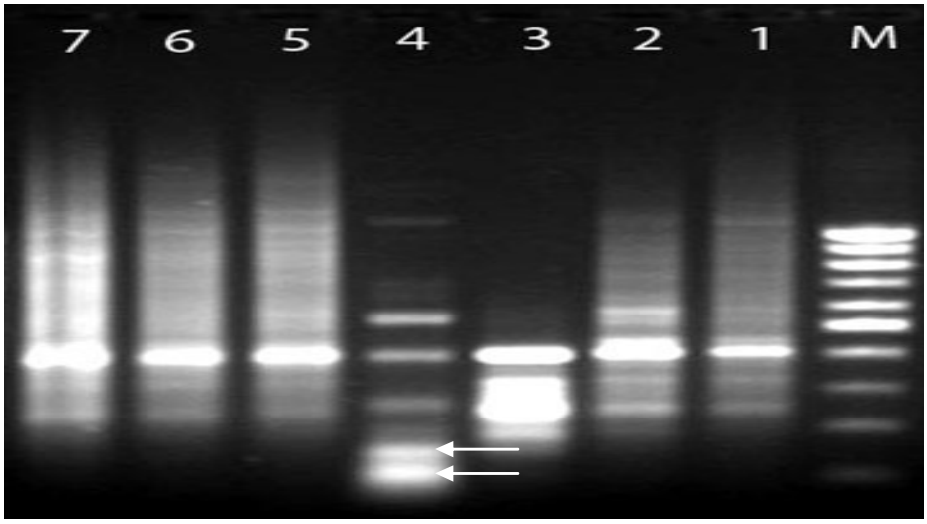

Figure 3. RAPD pattern of the *in vitro* preservation treatments of *D. scabra* with primer 3. M. DNA marker (100 - 1500 pb). (1) Mother plant; (2) Control; (3) Control + AC (4) 20 g/L mannitol; (5) 20 g/L mannitol + AC; (6) 40 g/L mannitol; (7) 40 g/l mannitol + AC.

mother plants were identical. While cultures conserved on mannitol treatments showed differences in DNA fragments than mother plants.

RAPD marker generated with primer (AM1) showed that the differences showed by three fragments; two fragments were detected at 1300 and 750 bp with 20 g/l mannitol with or without AC and 40 g/L mannitol without AC and one fragment was detected at 900 bp with 40 g/L mannitol with AC, which did not detected with the mother plants. RAPD marker generated with primers (AM2 and AM3) showed that the differences between the mother plants and mannitol treatments was found with 20 g/L mannitol as shown in primer AM8 two fragments at 500 and 600bp and in primer AM9 two fragments at 187 and

121 bp detected with 20 g/l mannitol without AC and was not detected along with the mother plants.

As RAPD markers amplify different regions of the genome, their simultaneous analyses gave a better interpretation of the genetic stability of the *in vitro* regenerates (Polambi and Damiano, 2002; Martins et al., 2004). Kovalchuk et al. (2008) reported that Preliminary RAPD analysis found no significant differences between apple germplasm plants stored for 39 months and non-stored controls. On the other hand, Harding (2004) demonstrated that the *in vitro* conservation of potato using tissue culture medium supplemented with the growth retardant mannitol causes morphological changes in the propagated material.

Conclusion

The present work confirmed that we can conserve *D. scabra* plants for 12 months in control medium (MS + 2 mg/L BA + 25 g/L sucrose + 7.0 g/L agar with or without AC) and incubated at 24°C. This medium could now be used for *in vitro D. scabra* culture, to improve management of large germplasm collections by reducing labor requirements and was found suitable to maintain the genetic stability with the mother plants.

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