Journal of Applied Horticulture, 26(1): 117-121, 2024
https://doi.org/10.37855/jah.2024.v26i01.22
Journal of Applied Horticulture
ISSN: 0972-1045

Micro propagation of non-seed setting hybrid of ornamental plant *Adenium obesum* (Forssk.) Roem. & Schult and genetic fidelity assessment using ISSR markers

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Abstract

Adenium obesum is a popular ornamental plant propagated through seeds. But its hybrids are mostly infertile or need assisted pollination for seed production. In the present investigation, an efficient and reliable indirect regeneration protocol for infertile adenium hybrid was developed from leaf explants. Surface sterilization using mercuric chloride 0.2 percent recorded the lowest incidence of contamination and highest survival percentage. Callus from shoot tip showed the lowest number of days for shoot regeneration with 13.2 days in Half-strength MS medium containing 3 mg L^{-1} NAA and 3mg L^{-1} GA_3. Maximum shoot length of 2.40 cm was recorded in Half-strength MS medium + 3 mg L^{-1} NAA+ 3mg L^{-1} GA_3 two weeks after sub culturing. Half-strength MS+2mg L^{-1} IBA recorded root initiation in 16.16 days. The rooted plantlets were successfully hardened and acclimatized with a survival rate of 92 %. The genetic fidelity of regenerated plantlets was assessed using ten primers and the *in vitro* cultured plants did not show polymorphism in ISSR analysis. This *in vitro* propagation protocol could be effectively used for the large-scale propagation of non-seed setting hybrids of adenium.

Key words Adenium, micropropagation, genetic fidelity, ISSR, tissue culture, desert rose, caudex, M S media

Introduction

Adenium obesum (Forssk.) Roem. & Schult, commonly known as the desert rose, belongs to the family Apocynaceae. These plants originated from the deserts of Africa, are scattered from Senegal to Ethiopia and from Somalia to Tanzania and are widely cultivated as ornamental plants in many humid and tropical countries. In their native environment, the plants are variable in appearance, slow growing and long-lived, surviving for hundreds of years. Its showy flowers of vivid colours, drought resistance and unique sculptural shaped caudex and roots has made it popular in the ornamental plant market (McBride *et al.*, 2014).

Ordinary varieties of adenium have light pink flowers with a reddish border on the petals, a thick light green caudex, and an average branching habit. But improved varieties have wide variations in flower colour and the number of petals. They have profuse flowering with long flowering duration, compactness, and dwarf habit. Apart from ornamental value, adenium has medicinal importance. It has a wide range of biological activities and exhibits anticancer, antiviral, antibacterial, trypanocidal, acaricidal, molluscicidal, antioxidant and piscicidal activities. All parts of the species are used in African traditional medicine (Paul *et al.*, 2015).

Common varieties of adenium are generally propagated through seeds and the plants originating from seeds exhibit more swollen caudex which is unique to adenium. However, not all adenium varieties produce seeds under cultivation conditions, since pollination is often unsuccessful due to male or female sterility (McLaughlin and Garofalo, 2002). Novel varieties of adenium are multi petal hybrids and many of them are sterile and some need assisted pollination for seed setting. Due to the absence of natural seed production, most of the hybrids require vegetative propagation methods like cuttings and grafting for multiplication. But these cannot be used as efficient methods for commercial production of adenium as they have low multiplication rate, and it requires many stock plants and skill. Vegetative propagation can produce only a limited number of plants due to the slow growth of the plants, which in turn leads to the high cost of adenium plants in the market. Considering these problems, micro propagation is the most cost-effective method for the large-scale production of non-seed setting hybrid adenium plants. Micro propagation has been reported in adenium using multiple shoots derived from *in vitro* grown seedlings by Kanchanapoom *et al.* (2010) and Rasad *et al.* (2015). But this method cannot be used in hybrids which fail to set seeds naturally. Hence, an alternative method using explants from field grown plants was standardized in the present study.

Genetic variations may appear in micro propagated plants due to numerous factors associated with *in vitro* culture conditions, especially clonal variations. Early assessment of genetic fidelity of micro propagated plants aids in fine-tuning the protocol parameters and to produce true to type plants. Therefore, an efficient detection tool like Inter Simple Sequence Repeats (ISSR) was used to ascertain the genetic fidelity of *in vitro* raised plants.

Material and methods

The explants *viz.*, shoot tip, nodal segments and leaves were collected from a non-seed setting field grown hybrid adenium plant. Explants were washed thoroughly in running tap water and rinsed in distilled water containing Tween 20 for about 5 minutes and kept under running tap water for 15 minutes. The

explants were kept at 0.1% Bavistin for 30 minutes and then washed under running tap water. Explants were then placed inside laminar air flow chamber and were surface sterilized with different concentrations of mercuric chloride (0.08, 0.1 and 0.2%) for 10 minutes and washed thrice using autoclaved distilled water. The surface sterilized explants were then immersed in 70% ethanol for about 30 seconds and washed thrice using double distilled water to remove the traces of $HgCl_2$.

The cut ends of the sterile explants were excised off and then made into small pieces in a sterile petri plate with sterile scalpel. The water droplets were removed by a sterilized blotting paper placed over a sterile petri dish. The explants were inoculated to Half strength MS (Murashige and Skoog, 1962) medium containing 30 g L^{-1} sucrose and 8 g L^{-1} agar with different concentrations of BAP (0, 0.5, 1.0, 1.5 mg L^{-1}) and NAA (0, 0.5, 1.0, 1.5 mg L^{-1}). The inoculated cultures were incubated in the culture room maintained at a temperature of 25 ± 2ºC with 40 µmol $m^{-2}s^{-2}$ intensity of light for 16 hour and 60% relative humidity. After every 20 days, the cultures were transferred to fresh medium containing BAP and NAA. For multiple shoot regeneration, well-proliferated callus was transferred to Half strength MS medium containing various concentrations of NAA (0, 1.0, 2.0 and 3.0 mg L^{-1}) and GA_3 (0, 1.0, 2.0 and 3.0 mg L^{-1}). The *in vitro* raised shoots (2.5 to 3.0 cm long) were excised and transferred to a Half-strength MS medium containing IAA (0, 1.0 and 2.0 mg L^{-1}) for *in vitro* rooting. The *in vitro* rooted plantlets were placed in cups containing a mixture of sterilized soil and sand in the ratio of 1:1. One month after planting, the well acclimatized plantlets were transferred to normal soil.

Three randomly selected plants and the mother plant were used for the analysis of genetic stability using ISSR markers. Leaf samples of 150 mg each of *in vitro* raised plantlets were used for the extraction of genomic DNA by Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1987). The quality and quantity of the DNA isolated were checked using Agarose (0.8 %) gel electrophoresis stained with Ethidium Bromide (0.5 μL^{-1}) and visualized in a UV transilluminator. The genetic stability of the *in vitro* raised plants was examined using ten ISSR markers.

PCR amplification was carried out using reaction mixture containing 5µL of DNA Polymerase 2X master mix, 2µL primer, 2µL of nuclease water and DNA 1µL with a total of 10µL reaction mix. PCR reaction was performed with an initial denaturation of DNA at 95 °C for 5 min, 34 cycles were performed each for 1 minute at 95ºC, 30 seconds at annealing temperature which is optimized and 1 minute 30 second extension at 72ºC, final extension step was carried out at 72ºC for 10 minutes. After the PCR, the amplified PCR products were electrophoresed on 1.2% agarose gel with 4µL of Ethidium Bromide. The running buffer used was 1X TAE and since the dye was already present in the master mix, the 10µL of the PCR products were directly loaded to the Agarose gel and the gel was run at 80V for 40 minutes. The gel was visualized, and bands were observed using gel documentation system.

Observations were recorded on various parameters namely number of successful cultures, days to callus initiation, number of successful callus cultures, days for shoot initiation, number of shoots per callus, number of nodes per shoot, shoot length, days for root initiation, number of cultures with root, number of roots per shoot and root length after 20 days of culture. All the results were expressed as mean ± standard error (SE) and the sample means were compared by using one-way ANOVA.

Results and discussion

Among the different concentrations of $HgCl_2$ used, the highest percentage of survival rate and lowest rate of contamination was recorded when the explant was surface sterilized with 0.2% $HgCl_2$ for 10 minutes. Shoot tip, leaves and nodal segments showed 48, 68 and 64% of survival respectively after two weeks of inoculation (Table 1). Plants harbor various bacteria and fungi while in the field. These microbes can cause systemic infection by penetrating the plant tissue. Thus, cultures derived from these tissues are susceptible to contamination (Chan and Evans, 1990). In the study, $HgCl_2$ (0.2%) treatment for 10 minutes was found to be most effective for surface sterilization. Similar results were also reported in adenium (Talukdar, 2012).

Table 1. Concentration of surface sterilization treatments on survival percent of explants

Survival (%)	Explant	$HgCl_2$ concentration		
		0.08%	0.10%	0.20%
After 1st week	ST	72	80	92
	L	80	84	100
	NS	80	88	92
After 2nd week	ST	48	64	72
	L	60	72	84
	NS	44	52	64
After 3rd week	ST	28	40	48
	L	36	48	68
	NS	32	44	64

*ST- Shoot tip, *L- Leaves, *NS- Nodal segments

Minimum number of days taken for callus initiation was obtained with leaf explants inoculated in the medium Half MS + 1.5 mg L^{-1} BAP +1.0 mg L^{-1} NAA. in 13.4 days. The maximum number of successful cultures were observed as 1.2 numbers in the same medium (Table 2). Rasad *et al.* (2015), obtained 100% callus induction when explants were taken from *in vitro* grown seedlings of *A. obesum* in the medium containing 1.5 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. According to Arumugam *et al.* (2009) variety of elements, such as culture, environment, the kind of explants, and hormonal and non-hormonal regulators, may work in concert to determine the correct induction, proliferation, and regeneration of callus into plants.

Among the three explants tried *viz*., shoot tip, leaves and nodal segments, the best explants were identified based on the number of successful cultures obtained. Culture establishment was found to be highest in the medium ½ MS + 1.5 mg L^{-1} BAP +1.0 mg L^{-1} NAA with an average of 1.8 and 2.0 numbers of successful cultures of shoot tips and leaves respectively. Selection of explants is a very important step in micro propagation to get high frequency of callus without contamination. In the present study, leaves and shoot tips showed culture establishment whereas no response was observed from nodal segments in all the media tried. Culture establishment was found to be highest in the medium Half MS + 1.5 mg L^{-1} BAP +1.0 mg L^{-1} NAA. Colombo *et al.* (2018), reported high frequency of callus initiation in MS medium

with 2.0 mg L^{-1} BAP and 1.0 mg L^{-1} NAA in shoot tip and leaf explants of *A. obesum*. Nodal segments showed browning of the nodes after two weeks of inoculation. This might be due to the latex exudation from the nodal segments.

The callus obtained were cultured in medium with different concentrations of NAA (0, 1.0, 2.0 and 3.0 mg L^{-1}) and GA_3 (0, 1.0, 2.0 and 3.0 mg L^{-1}) to study its effect on shoot regeneration. Callus from shoot tip showed the lowest number of days for shoot regeneration with an average of about 13.2 days in the medium Half MS + 3 mg L^{-1} NAA+ 3 mg L^{-1} GA_3. Maximum shoot length was recorded with an average of 2.40 cm in the medium ½ MS + 3 mg L^{-1} NAA+ 3 mg L^{-1} GA_3 two weeks after sub culturing (Table 3).

Table 2. The effect of different concentrations of BAP and NAA on callus initiation and culture establishment

Treatment	Number of days for callus initiation	Number of callus cultures obtained from shoot tip explant	Number of callus cultures obtained from leaf explant
Half MS (Control)	19.4 ± 0.894[a]	0.6 ± 0.894	0.6 ± 0.894
Half MS + 0.5 mg L^{-1} BAP +0.5 mg L^{-1} NAA	17.4 ± 0.894[b]	0.8 ± 1.304	1.2 ± 1.304
Half MS + 1.0 mg L^{-1} BAP +0.5 mg L^{-1} NAA	16.4 ± 0.894[bc]	0.8 ± 0.837	0.6 ± 0.894
Half MS + 1.5 mg L^{-1} BAP +1.0 mg L^{-1} NAA	13.4 ± 0.837[c]	1.8 ±1.304	2.0 ± 1.584
Half MS + 1.0 mg L^{-1} BAP +1.5 mg L^{-1} NAA	15.6 ± 1.10[d]	0.6 ±0.548	1.2 ± 1.304

Values represent means ± Standard Error. Means followed by the same alphabets within each column are not significantly different (P ≤ 0.05) according to Duncan's Multiple Range Test

No shoot regeneration was observed in the callus from leaves and browning was observed. Among the different concentrations of NAA and GA_3 (0, 1, 2 and 3 mg L^{-1}), 3 mg L^{-1} of NAA and GA_3 in the Half strength MS medium showed maximum number of shoots with the least number of days when compared with other concentrations. The results obtained in this study is similar to the investigation done by Colombo *et al.* (2018), where callus obtained from leaf explants were unable to induce shoot regeneration while callus from shoot tips did.

The *in vitro* cultured shoots were separated and cultured in Half strength MS medium containing different concentrations of IBA (0, 1.0 and 2.0 mg L^{-1}). The Lowest number of days taken for root initiation was observed in the medium Half MS + 2.0 mg L^{-1} IBA in 16.16 days. The maximum number of roots per shoot was 9.83. The highest root length of 3 cm was recorded in 2.0 mg L^{-1} IBA (Table 3). Maximum rooting and root length were observed in the medium supplemented with 2.0 mg L^{-1} IBA. Similar results were observed by Azad and Amin (2013), who reported that IBA was comparatively more effective than NAA and IAA for *in vitro* rooting in *Muscari armeniacum.*

Table 3. The effect of different concentrations of growth regulators on multiplication, shoot regeneration and root induction of *in vitro* propagated plants of *Adenium obesum*

Concentration of growth regulator (mgL^{-1})	Days to shoot initiation	Average number of shoots per callus	Average number of nodes per shoot	Average shoot length (cm)
Half MS	19.0 ±1.00a	0.8 ±0.837	0.8 ±0.447	1.08 ±0.239
Half MS + 1.0 mgL^{-1}NAA +1.0 $mgL^{-1}GA_3$	18.0 ±0.707a	1.0 ±0.707	1.0 ±0.00	1.20 ±0.212
Half MS + 2.0 mgL^{-1}NAA +2.0 $mgL^{-1}GA_3$	16.8 ±0.837b	0.8 ±0.447	1.0 ±0.00	1.34 ±0.385
Half MS + 3.0 mgL^{-1}NAA +3.0 $mgL^{-1}GA_3$	13.2 ±0.837c	1.0 ±0.00	1.0 ±0.00	2.40 ±0.962
Concentration of growth regulator (mgL^{-1})	Days to root initiation	Average number of cultures with root	Average number of roots per shoot	Average Root length (cm)
Half MS	20.500± 1.049b	0.667± 0.516a	0.667± 0.516b	0.667± 0.408b
Half MS + 1 mgL^{-1}IBA	18.500± 1.049c	2.333± 1.033b	4.167± 1.472c	1.667± 0.816bc
Half MS + 2 mgL^{-1}IBA	16.167± 1.169a	2.333± 1.211a	9.833± 2.041a	3.000± 1.414a

Values represent means ± Standard Error. Means followed by the same alphabets within each column are not significantly different (P ≤ 0.05) according to Duncan's Multiple Range Test

The well rooted plantlets obtained on Half strength MS medium with 3 mg L^{-1} NAA and 3 mg L^{-1} GA were further used for acclimatization. Plantlets were carefully taken out of the medium and washed thoroughly in running tap water to remove all traces

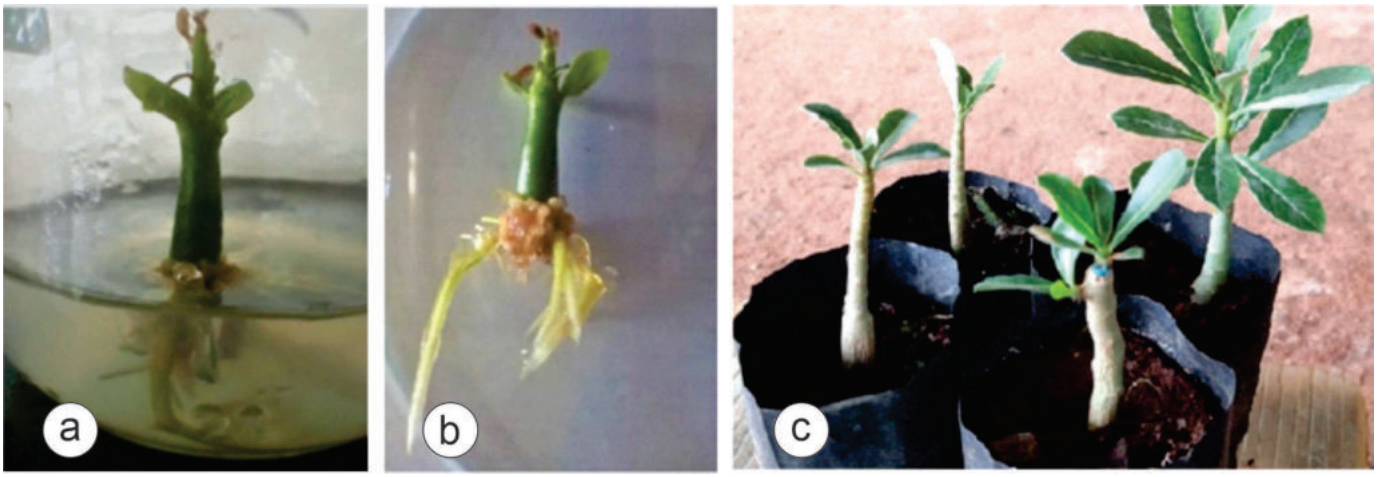

Fig.1. a and b *in vitro* rooted plants, c acclimatized plants

of medium attached. Plantlets were planted in cups with sterilized soil and sand in the ratio of 1:1(Fig.1). The cups were covered with polythene bags to maintain humidity and kept in an indoor growing room. All these plantlets were kept under similar and controlled environmental conditions. The temperature and relative humidity of growing room was maintained at 25-30°C and 70%, respectively. The plants were regularly watered to ensure that the pots retained an adequate level of moisture. The plantlets were gradually acclimatized to the field condition with a survival percentage of 92% after two months. Three healthy plants were randomly chosen from a total of 40 micropropagated and hardened plants. The genetic stability of these selected plants was then analyzed using ISSR markers, with their respective mother plant serving as the basis for comparison.

The ten ISSR primers screened for the genetic fidelity assessment, generated a total of 52 bands by HB-12, DDS-102, DDS-104, UBC-818, UBC-825, UBC-827, UBC-868 and UBC-880. Primers DDS-101 and DDS-103 showed no amplification (Table 4).

The continuous process of subculturing carries the risk of introducing genetic variability, particularly through somaclonal variations. Factors such as the culture method, conditions, source and developmental stage of the explant, ploidy level, and frequency of subculture significantly influence the occurrence of these variations. It is critical to carefully evaluate the suitability of micro-propagation protocols tailored to each species to ensure genetic uniformity in micro-propagated plants. This uniformity is essential for achieving commercial success, relying on maintaining clonal consistency.

Table 4. ISSR markers screened for the genetic fidelity assessment

Sl. No.	Primers	Sequence (5'-3')	Remarks
1	HB-12	CACCACCACGC	Monomorphic
2	DDS-101	AGGAGGAGGAGGAGGAGG	No amplification
3	DDS-102	GAGAGAGAGAGAGAGAGAT	Monomorphic
4	DDS-103	GACAGACAGACAGACA	No amplification
5	DDS-104	TGAGAGAGAGAGAGAGAGA	Monomorphic
6	UBC-818	CACACACACACACACAG	Monomorphic
7	UBC-825	ACACACACACACACACT	Monomorphic
8	UBC-827	ACACACACACACACACG	Monomorphic
9	UBC-868	GAAGAAGAAGAAGAAGAA	Monomorphic
10	UBC-880	GGAGAGGAGAGGAGA	Monomorphic

Various methodologies, including morphological descriptions, physiological observations, cytological studies, field assessments, isozyme analysis, and molecular studies, have been developed to assess the genetic purity of tissue-cultured plants. RAPDs and ISSRs are widely used molecular markers due to their simplicity, speed, cost-effectiveness, and high discriminatory power. ISSR primers, with their longer sequences, offer advantages in detecting somaclonal variations, demonstrating higher sensitivity, stringent reproducibility, and dominant representation of polymorphic alleles (Solanke *et al.*, 2017).

In the present study (Fig. 2), no ISSR polymorphisms were detected in the plants, indicating genetic stability compared to the mother plant. The banding patterns observed in both the in vitro cultured plants and the mother plant were identical, confirming genetic stability in the cultured plants. The results of this study corroborate with the findings of Joshi and Dhawan (2007) and Faisal *et al.* (2012) where ISSR markers exhibited bands for *in vitro* raised plants when compared with mother plants.

In the present study, culture establishment of *A. obesum* was successful from shoot tip explants surface sterilized in 0.2% $HgCl_2$ for 10 minutes and cultured in Half MS + 1.5 mg L^{-1}

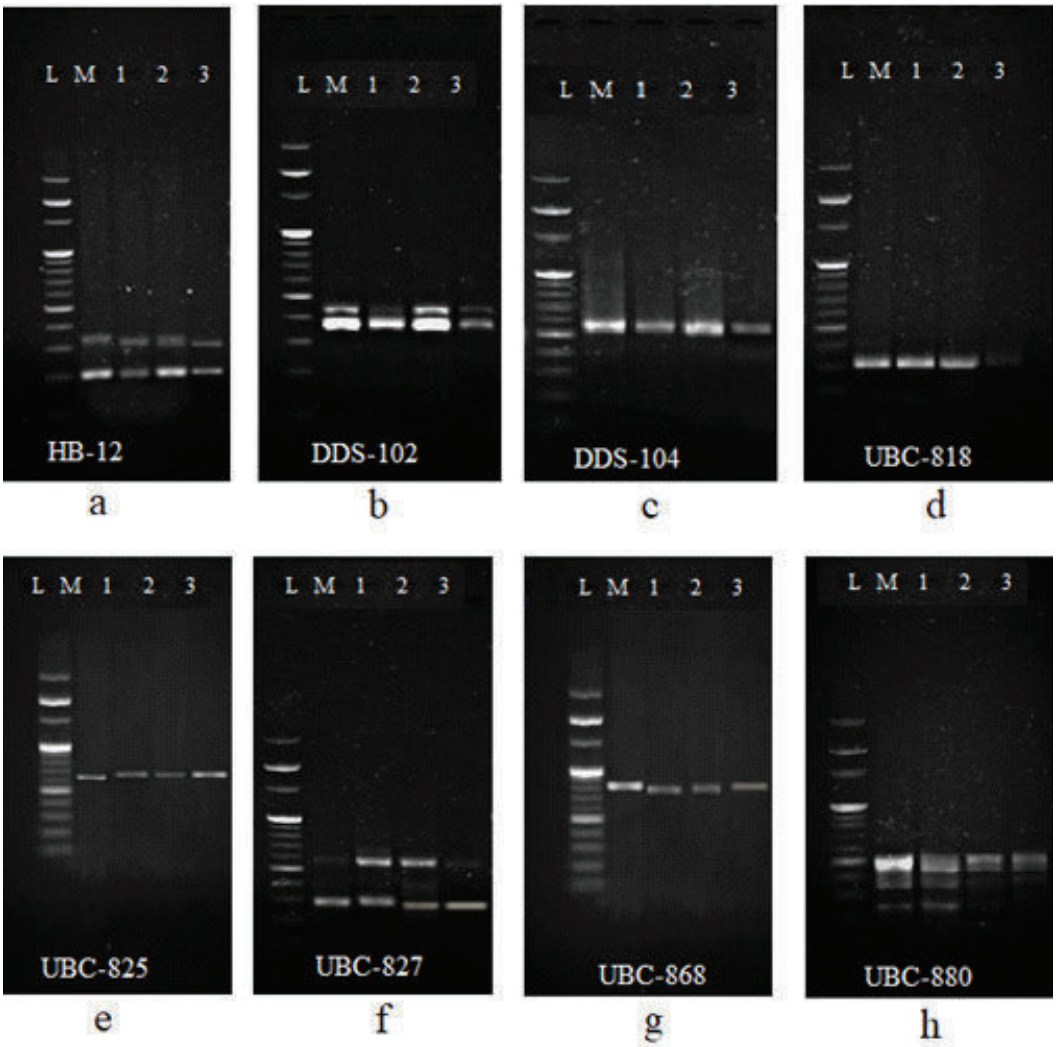

Fig. 2. Inter-simple sequence repeats (ISSR) amplification pattern obtained for mother plant (M) and daughter plant of 1, 2 and 3 of *Adenium obesum*; using (a) primer HB-12, (b) primer DDS-102, (c) primer DDS-104, (d) primer UBC-818, (e) primer UBC-825, (f) primer UBC-827, (g) primer UBC-868 and (h) primer UBC-880

BAP + 1mg L^{-1} NAA at a temperature of 25 ± 2ºC for 16 hours photo period. Organogenesis could be induced in the medium Half MS with 3 mg L^{-1} NAA and 3 mg L^{-1} GA. Based on our results, using ISSR markers, the number of the primers (10) used in this study, as well as total number of bands (52) together with the observed homogeneity of the plants generated in this study, strongly suggest that the regeneration from shoot tip explants is a reliable approach for propagation of hybrid *A. obesum* which fail to set seeds naturally. Being a popular ornamental plant, these results will help in the large-scale production of non-seed setting adeniums.

Conflict of interest: All Authors read, approved the manuscript, and declare that they have no conflict of interest.

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Received: October, 2023; Revised: December, 2023; Accepted: January, 2024