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In vitro Propagation of *Adenium obesum* (Forssk.) Roem. and Schult.

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Abstract

An *in vitro* protocol using shoot tip explants from seedling of *Adenium obesum* (Forssk.) Roem. and Schult. was developed. Explants were cultured on Murashige and Skoog (MS) medium supplemented with various combinations of NAA and BA. The most effective medium for shoot proliferation at a high frequency of 5.20±1.10 shoots per explant consisted of 22.2 μM BA. High rooting and survival were achieved using MS medium supplemented with 0.3% activated charcoal and without any growth regulators. Rooted plants were successfully acclimatized to greenhouse conditions. This study showed that *A. obesum* could be micropropagated by utilizing multiple shoots derived from seedling shoot tips. A flow cytometric analysis for DNA content revealed no differences among the micropropagated plants and the *in vivo* natural grown plants. The resulting 2C DNA value (8.35±0.039 pg) of this species was estimated for the first time.

Keywords: desert rose, flow cytometry, nuclear DNA content, shoot tip culture

Introduction

Adenium obesum (Forssk.) Roem. and Schult., also known as desert rose, belongs to the family *Apocynaceae*. It is native to the East Africa and has been a very popular ornamental plant for decades. *A. obesum* is a beautiful succulent shrub with a stout swollen stem, grayish bark, and striking pink flowers. In Thailand, the interest for growing it as a pot plant has been increasing markedly as a result of an increasing demand for landscape and indoor decoration (Wannakrairoj, 2008). *A. obesum* can be propagated by seeds, cuttings or transplants. Cutting is an inefficient propagation method for pot plant, since the planting material has a very low multiplication rate and it requires a large area of stock plants. Thus, micropropagation seems to be the most promising method for large-scale production of plantlets for use as pot plants. Seedlings germinated from seed culture can be used as explants for micropropagation. The other advantages of clonal propagation over conventional propagation techniques include: true to type, rapid multiplication, and lower production cost. However, no information has been reported on the *in vitro* regeneration of this potted plant.

It is axiomatic that plants regenerated from well-developed meristematic tissue, show the lowest tendency of genetic variation (Rout *et al.,* 1998). This variation can be altered through changes either in the chromosome number or in the ploidy level. Therefore the analysis of *in vitro* regenerated plants by measuring cell nuclei DNA content using flow cytometry is of particular importance. Flow cytometry was used in several studies of ornamentals to estimate the nuclear DNA content such as *Plantago asiatica* (Makowezyńska *et al.,* 2008), *Alocasia micholitziana* (Phuong Thao *et al.,* 2003), *Dendrobium crumenatum* (Meesawat *et al.,* 2008), *Ranunculus asiaticus* (Dhooghe *et al.,* 2009). In this context, the present communication describes the development of a micropropagation protocol using shoot tips derived from aseptically grown seedlings of *A. obesum*. Consequently, nuclear DNA content of both micropropagated and natural grown plants was analyzed as well.

Materials and methods

Plant materials

Seeds of *A. obesum* were obtained from a commercial company. They were surface sterilized using 95% ethanol for 1-2 min and then immersed in 10% (v/v) Clorox™ solution (active chlorine 0.5%) containing 2 drops of Tween-20 emulsifier per 100 ml solution for 30 min. The sterilized seeds were washed three times with sterile distilled water to remove traces of disinfectant and sown on Murashige and Skoog (MS)(1962) basal medium. After 3 weeks of culture, seedlings with well develop shoots and roots were obtained.

Culture media

Shoot tips of 3 week-old seedlings were used for multiplication experiments. Shoot tips were cut about 0.5-1 cm above the stem of seedlings. These explants (1 cm long) were excised aseptically and cultured on MS basal medium supplemented with α-naphthaleneacetic acid (NAA) at the concentrations of 0, 2.7 or 5.4 μM and N^6-benzyladenine (BA) at the concentrations of 0, 4.4, 13.3 or

22.2 μM either singly or in combinations. A culture cycle was 4 weeks. Following this period, the plants were transferred to a fresh medium or used for root induction. To establish root proliferation, individual isolated shoots (1.5 cm long) from shoot multiplication cultures were excised and placed on MS medium supplemented with 0, 4.9, 9.8, 14.8, or 19.7 μM indole-3-butyric acid (IBA) or without IBA and 0.3% activated charcoal (AC).

Culture conditions

All media were supplemented with 30 g l^{-1} sucrose and 8.2 g l^{-1} of Mermaid™ agar. The pH of all media was adjusted to 5.7 with 1 N NaOH or 1 N HCl prior to autoclaving at 1.05 kg/cm^2, 121˚C for 20 min. Cultures were maintained at 25±1˚C air temperatures in a culture room with a 16/8 h light/dark photoperiod under an illumination of 20 $\mu molm^{-2}s^{-1}$ photosynthetic photon flux intensity provided by cool-white fluorescent light. Plant materials were stored in glass-capped culture jars (115 ml capacity) each containing 20 ml of medium.

Flow cytometry analysis

Nuclear suspensions from 1year-old micropropagated and mother plant leaves were prepared according to Otto (1990). Briefly, nuclei were released from leaf cells (typically 20 mg) by chopping the tissue with a razor blade in a Petri dish on top of ice-cold bucket containing 1 cm^3 of Tris-$MgCl_2$ buffer (0.2 M Tris, 4 mM $MgCl_2$, 0.5% (w/v) Triton X-100, pH 7.5) (Pfosser *et al.,* 1995). The suspension of nuclei was then filtered through a 42 μm nylon mesh filter and 50 μl each of Propidium iodide (50 μg l^{-1}) and RNAse (50 μg l^{-1}) were added to the samples to stain DNA. Samples were analyzed within 15 min period in a flow cytometer FACScalibur Becton Dickinson. Leaves of *Zea mays* CE-777 (2C=5.43 pg DNA), an internal reference standard, was kindly provided by Dr Jaroslav Dolezel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Czech Republic. The fluorescence of at least 5000 Propidium iodide-stained nuclei was analyzed per sample. The relative nuclear DNA content of 10 micropropagated plantlets was estimated and compared with the values obtained from 10 replicates of mother plants grown in the green house. The reference standard peak was set to show at channel 200 of relative fluorescence intensity. The obtained histograms were computerized by Cell Quest software packages (Becton Dickinson, USA).

Plant acclimatization

Regenerated shoots were transferred to the basal MS medium supplemented with 0.3% AC to promote root elongation. Mermaid™ agar was carefully washed from the plantlets and they were then transferred to black plastic pots filled with sterile vermiculite until root systems were well established. These plantlets were watered twice daily. Later they were transplanted in a potting mixture of sand, manure and decayed leaf (1:1:1) and hardened in a mist bed for 1 month.

Statistical analysis

One explant was planted per jar and all experiments were carried out with 20 replicates per treatment and the experiments were repeated on consecutive days. Number of shoots and roots were evaluated after each culture period and submitted to analyze the variance. Mean of numbers were compared using Duncan's multiple range test at $p \leq 0.05$. The software used was SPSS for Windows XP Professional.

Results and discussion

The effect of NAA and BA on shoot multiplication is presented (Tab.1, Fig. 1). The percentage of explants developing shoots was 100% and multiple shoots were formed at a high frequency of 5.20±1.10 on MS medium containing 22.2 μM BA. The leaves of the adventitious shoots were healthy with green color and did not show any sign of vitrification (Fig. 2a).

Although both BA and NAA induced shoot multiplication in *A. obesum*, differences in shoot regeneration were evidenced. The presence of BA alone in the medium appeared to be optimal for shoot induction. This may suggest that bud formation required cytokinin. A combination of BA and NAA did not evoke a better response in shoot multiplication than BA alone. This is probably due to the difference endogenous levels of growth regulators in this plant or to a difference in sensitivity (Trewawas and Cleland, 1983). In *Dianthus chinensis*, high efficiency shoot bud formation and plant regeneration were achieved when the culture medium contained both BA and NAA

Tab. 1. Effect of different combinations of NAA and BA on shoot multiplication in *A. obesum* shoot tip explants cultured on MS medium

NAA (μM)	BA (μM)	Shoot induction (%)	No. of shoots per shoot tip (Mean±SE)
0	0	100	1.00±1.12[b]
0	4.4	100	4.20±2.28[a]
0	13.3	100	4.60±1.82[a]
0	22.2	100	5.20±1.10[a]
2.7	0	100	1.10±0.00[b]
2.7	4.4	100	2.40±1.67[b]
2.7	13.3	100	3.20±2.05[a]
2.7	22.2	100	4.40±0.89[a]
5.4	0	100	3.00±1.58[a]
5.4	4.4	100	2.40±0.89[b]
5.4	13.3	100	2.00±0.71[b]
5.4	22.2	100	2.60±1.67[b]

The different letters within column show significant difference of shoot number (Mean±SE) analyzed by Duncan's multiple range test at $p \leq 0.05$.

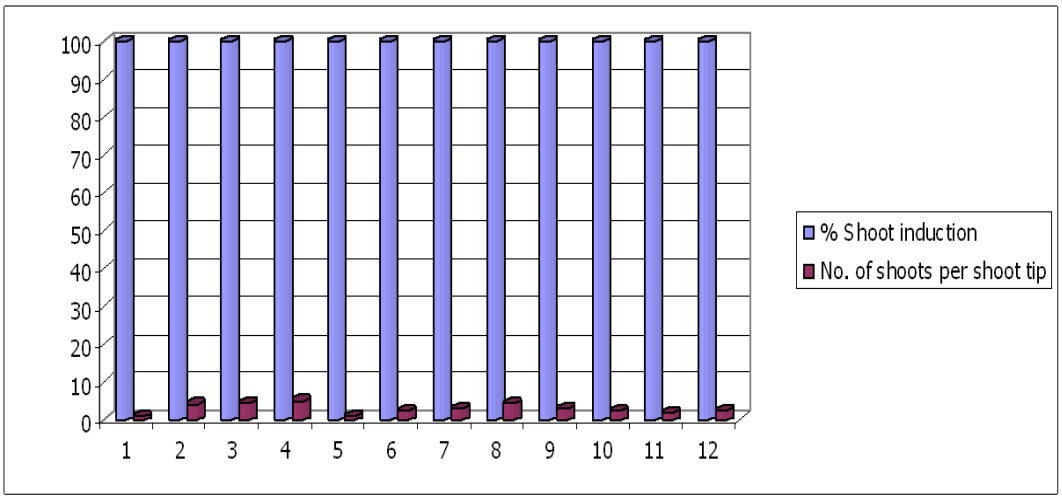

Fig. 1. Effect of different combinations of NAA and BA (1-12) on shoot multiplication in *A. obesum* shoot tip explants cultured on MS medium

(Kantia and Kothari, 2002). In *A. obesum*, application of NAA solely had little effect on shoot bud induction compared to BA alone. There was a considerable increase in shoot number on lower NAA (2.7 µM) and higher BA levels (4.4-22.2 µM). It can be inferred from the above results that shoot multiplication of *A. obesum* was easily achieved for all BA and NAA treatments and this suggests that *A. obesum* is amenable to *in vitro* propagation. This is in concurrence with the results in other ornamental plants such as *Tagetes* (Kothari and Chandra, 1984; Belarmino, 1992), *Lilium* (Liu, 1986) and *Dianthus* (Jethwani and Kothari, 1993). Therefore MS medium containing 22.2 µM BA

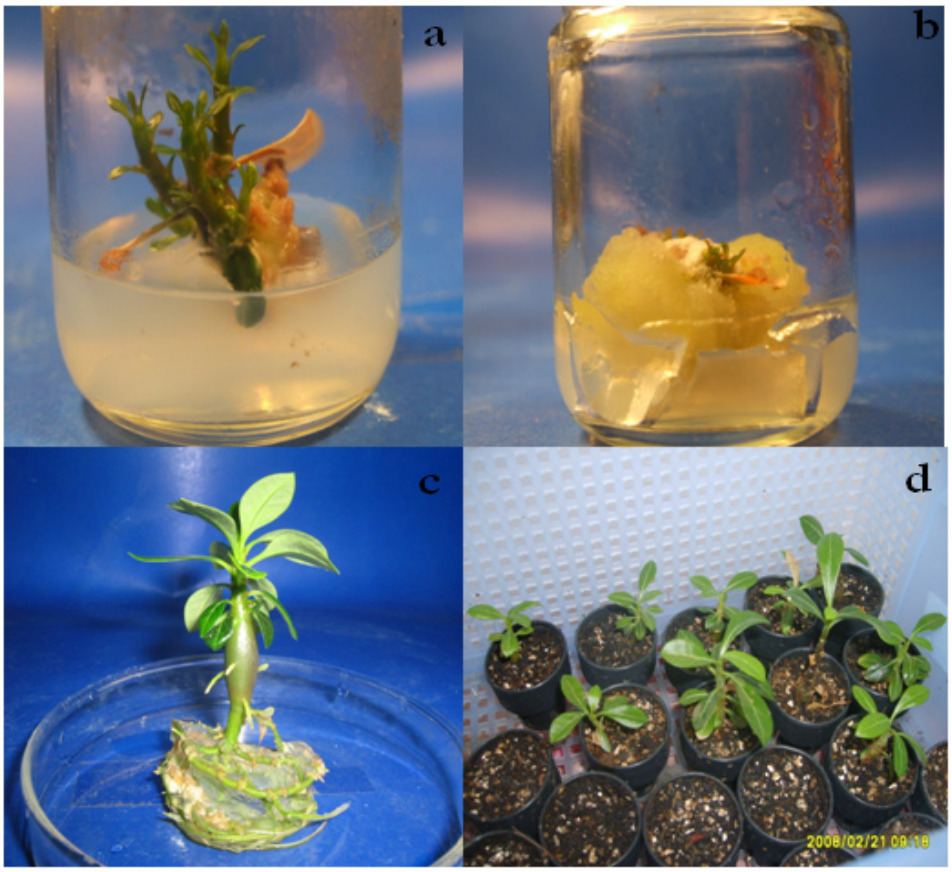

Fig. 2. Micropropagation of *A. obesum*. (a); Multiple shoot formation from shoot tip culture; (b) Callus formation; (c) Rooted plantlet,;(d) Three-month-old plantlets

Tab. 2 Effect of different combinations IBA and AC on rooting of *A. obesum* shoots

IBA (µM)	AC (%)	Root induction (%)	No. of roots per shoot (Mean ± SE)
0	0.3	99.2 ± 1.5[a]	12.4 ± 1.9[a]
4.9	0	80.5 ± 1.3[b]	4.40 ± 1.3[b]
9.8	0	77.6 ± 2.0[b]	1.40 ± 2.0[b]
14.8	0	75.4 ± 1.4[b]	1.87 ± 1.4[b]
19.7	0	79.1 ± 1.2[b]	2.00 ± 1.2[b]

The different letters within column show significant difference of root induction and root number analyzed by Duncan's multiple range test at $p \leq 0.05$.

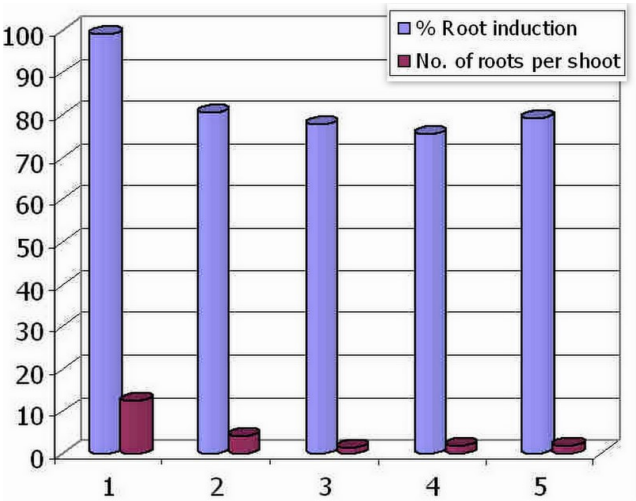

Fig. 3. Effect of different combinations of IBA and AC (1-5) on rooting of *A. obesum* shoots

was considered as optimal for shoot proliferation and the *in vitro* regenerated shoots with green expanded leaves and single main stem were further multiplied by subculturing 1.5 cm long segments individually onto this medium. These multiple shoots continued to proliferate after several subcultures with an average of 5 shoots per cycle.

With NAA and BA at all concentrations incorporated in the media resulted in yellow friable callus formation. In the beginning, callus started from the cut end of explants and growth was rather slow and after a 4 week subculture period the speed of callus proliferation was fast (Fig. 2b). Attempts to develop an efficient system to regenerate via callus culture are a prelude for genetic transformation system in *A. obesum.*

The induction of roots is an important procedure to form the complete plantlets. Regenerated shoots obtained from the shoot induction media did not produce roots therefore single shoots (1.5-2 cm long) were excised and used for *in vitro* rooting. The effect of IBA and AC on rooting is presented (Tab. 2, Fig. 3). MS medium supplemented with IBA was effective in terms of production of roots per shoot. With 4.9 µM IBA incorporated in the medium,

Tab. 3. Mean DNA content in picograms for A. obesum leaves from natural and in vitro grown plant

A. obesum	DNA content (pg)±SE
Natural grown plant	8.35±0.039[a]
In vitro grown plant	8.31±0.021[a]

The different letters within column show significant difference of DNA content (Mean±SE) analyzed by Duncan's multiple range test at $p \leq 0.05$

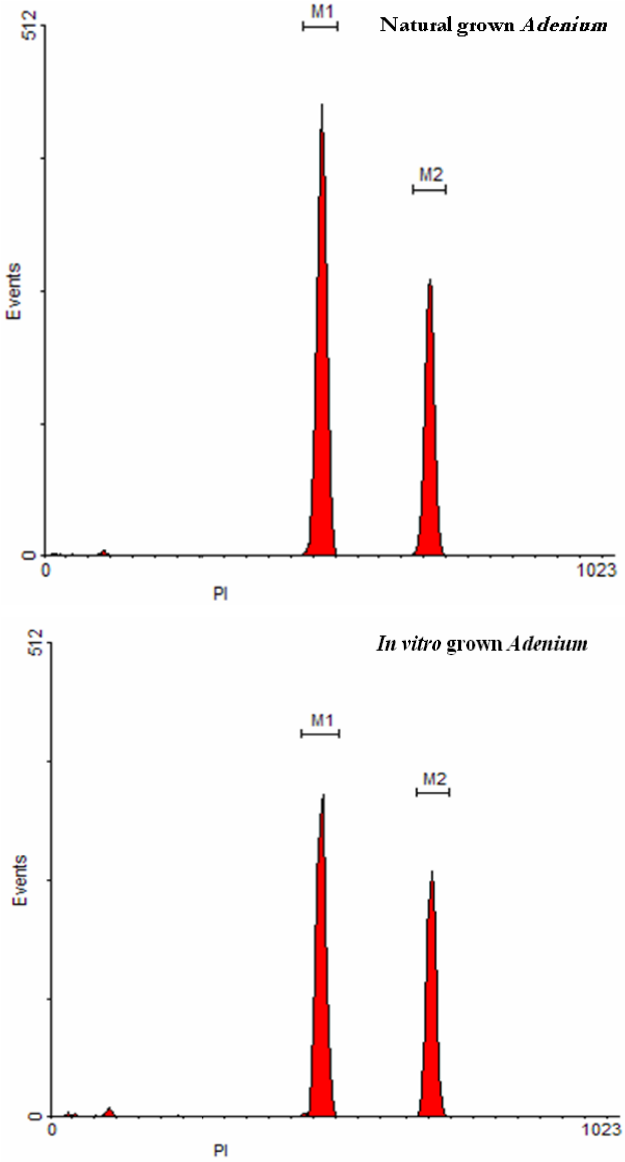

Fig. 4. Histogram of fluorescence intensity of *A. obesum* nuclei stained with Propidium iodide. Results represent measurements of *Zea mays* CE-777 (M1) and *A. obesum* (M2)

numerous thin and long roots (4.40±1.34) were obtained. However, the highest rooting number (12.4±1.98) with large and vigorous root system was observed on MS medium devoid of growth regulators but with 0.3% AC (Fig. 2c). Up to 99% rooting was achieved on this medium. This result revealed that *A. obesum* did not require the presence of auxins in the medium for root induction. Similar result was observed in *Tagetes mendocina* (Benavides and

Caso, 1993). AC may adsorb waste exudates released by explants or create partial darkness which is similar to the underground environment thus causes the increased rooting. When the complete plantlets reach a length of 6-7 cm, agar was carefully washed from these plantlets. They were transferred to small black plastic pots containing sterile vermiculite (Fig. 2d). The percentage of survival plantlets was 70% in a nursery under shade and natural photoperiod. Normal looking *A. obesum* plantlets thus obtained did not display any phenotypic variation during subsequent vegetative development.

Flow cytometry is an efficient method for the rapid detection of DNA content and ploidy level variation (Dolezel, 1991). Determination of DNA content has been previously reported in other ornamental plant species such as *Dendrobium* (Jones *et al.,* 1998). The present study presents 2C DNA content of *A. obesum* as determined by flow cytometry. Alteration of DNA content was not observed among micropropagated plants and natural grown plants. Flow cytometry analysis revealed mean 2C DNA contents were 8.31±0.021 pg $2C^{-1}$ for in *vitro* grown plants and 8.35±0.039 pg $2C^{-1}$ for natural grown plants (Tab. 3, Fig. 4).

Conclusions

In conclusion a system to regenerate *A. obesum* through multiple shoots from shoot tip of seedling is established for the first time. Shoot tip appeared to be a potentially suitable explant for micropropagation thus makes it well-suited for large scale commercial propagation. The analysis of nuclear DNA content of *A. obesum* was also estimated using flow cytometry (2C=8.35±0.039 pg).

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