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Research Article

IN VITRO REGENERATION OF AN ENDANGERED ORNAMENTAL PLANT IMPALA LILY (*ADENIUM MULTIFLORUM* KLOTZSCH)

***Tulika Talukdar**
**Department of Botany, Krishnagar Govt. College, University of Kalyani, Krishnagar, 741101,West Bengal, India*
**Author for Correspondence*

ABSTRACT
Adenium multiflorum Klotzsch is an endangered ornamental plant belonging to the family Apocynaceae. Poor seed germinability and very low multiplication rate of planting materials pose high threat to this pot plant, and its difficult propagation makes conservation essential. The present investigation is an effort to establish *Adenium* for micro-propagation and sub-culturing as a rapid, cost-effective alternative tool. Juvenile leaves were used as explants for *in vitro* organogenesis through callusing and cultured on three different basal media-MS, B-5 and N_6 supplemented with phytohormones -α-naphthaleneacetic acid (NAA), kinetin and Gibberelic Acid (GA). Regenerated shoots were transferred to half strength basal media (MS, B-5 and N_6) supplemented with 2% (w/v) sucrose, 0.8% difcobactoagar and different concentrations of growth regulators - NAA and kinetin for 1-2 weeks. The regenerated shoots were also tested on growth regulator free half strength basal media for rooting. The complete rooted plantlets were initially acclimatized in moist chamber and finally under semi-shady condition. This is the first report of complete regeneration of *A. multiflorum* from callus via shoot organogenesis.

Key Words: *Adenium Multiflorum Klotzsch, In Vitro, Ornamental, Regeneration, Basal Medium, PGRS*

INTRODUCTION
Adenium multiflorum Klotzsch, also known as Impala Lily, is a beautiful ornamental plant in the family Apocynaceae. Due to its 'bon-sai' like appearance and flowers having brilliant combinations of red, white, pink and crimson coloration *A. multiflorum* has gained tremendous popularity as an ornamental cash crop in domestic (India) as well as in international market. It is native to tropical Africa and Arabia, but introduced and naturalized in different parts of the world including South-east Asia (Oyen 2008). The plant, recently, has been enlisted in Red Data Lists of several tropical African countries where it is regarded as threatened. Habitat destruction, over-exploitation for medicinal and illegal horticultural trade, and poor propagation in its natural habitat are the main threats to Adeniums. In India, the interest for growing it as a pot plant has been increasing markedly due to its rising demand for landscape and indoor decoration (Bhattacharjee 2006). *A. multiflorum* can be propagated by seeds, cuttings or transplants. However, both are inefficient propagation methods for the pot plant, since the germinability of seed is very poor and the planting material not only has a very low multiplication rate but it requires a large area of stock plants also (Anderson 1983; Kanchanapoom *et al*., 2010). *In Vitro* regeneration technique has emerged as a feasible and cost-effective alternative tool for rapid production of ornamentals in recent years. In a developing economy like India, the market for ornamentals has steadily increased over the last few years (Hossain *et al*., 2007) and Adeniums has good opportunity to occupy a significant share of this nascent market.

The development of a reliable *in vitro* protocol is also of great importance for conservation of rare and endangered plant species. Furthermore, stress tolerant lines exhibiting high antioxidant defense activities can be effectively screened through this technique as also practiced in different crops through induced mutagenesis (Hossain *et al*., 2007; Talukdar 2011, 2012). Plant regeneration from tissue cultures of many plant species has been reported, but some groups, families, and genera are still regarded as recalcitrant (Ochatt *et al*., 2010). No information has been reported on the *in vitro* regeneration of potted *A. multiflorum* plant so far. In the present context, main objective of the study was to establish an efficient

protocol to regenerate *A. multiflorum* plants via callus culture to bypass the dormancy in the seeds and also to meet growing demand of *A. multiflorum* in floriculture trade.

MATERIALS AND METHODS

Plant Material

The plant of *A. multiflorum* grown in earthen pot was used as an experimental donor plant. Juvenile leaves from 3 months old donor plant were used as explants. Leaf explants were washed thoroughly under running tap water for 30 min and for another 10 min with 5% (v/v) aqueous solution of Teepol, followed by washing with single distilled water three times. Teepol, a liquid detergent was used to remove the dust and dirt particles from the leaf surface. Leaves were then treated with bavistin (a systemic fungicide) solution for 4-5 min, followed by rinsing thoroughly with sterile distilled water. The leaf explants were then quickly dipped (for 30 sec) in 70% ethanol and surface sterilized with 0.2% (w/v) aqueous $HgCl_2$ solution for 2 min, followed by repeated washing with sterile distilled water. All leaves were dissected into small pieces (approximately 0.5-0.75 cm length) and trimmed, so that maximum part can be exposed to media.

Culture Media and Growth Conditions

To search an efficient medium, regeneration potential of three different basal media namely Murashige and Skoog (1962), Gamborg' B5 (Gamborg *et al.*, 1968) and N_6 (Chu *et al.*, 1975) were used, each fortified with 3% (w/v) sucrose and 0.8% Difco-bactoagar. The plant growth regulators (PGRs) used were α –naphthalene acetic acid (NAA), kinetin, and GA. The pH of the medium was adjusted to 5.6-6.0 prior to autoclaving at 121 $^{\circ}$C for 15 min. All the experiments were carried out in culture tubes (150x25 mm) containing 25-30 mL of culture medium. Temperature of the culture room was maintained at 25 ± 1 $^{\circ}$C under a 16-h photoperiod with a light intensity of 3000 lux and relative humidity of 65-70%.

Callus Induction and Establishment

For callus induction juvenile leaf section (approximately 0.5 cm long) with cut end surface was placed in contact with culture medium supplemented with growth regulators. Each of the basal media was supplemented with various concentration of PGRs- NAA, kinetin and GA separately. After minimum 20 days of culture, the leaf explants were found to give callusing. The calli of different media and of different PGR concentrations were subcultured after every 10-15 days for successive three times in the same media composition. Days to callus initiation, percentage of callus induction and subsequent growth rate of calli [expressed in % of fresh mass (FM) increased] during subculturing were recorded separately for different media and different PGR concentrations. Minimum 3 replicates were maintained for each treatment.

Shoot Organogenesis

For shoot organogenesis, well growing green calli (Figure 1a) were inoculated on same basal media (MS, B5 and N_6) containing 3% sucrose, 0.8% difcobactoagar and fortified with NAA and kinetin in different concentrations. The pH adjustment of medium and autoclaving were done as earlier. All the cultures were incubated at 25 ± 1 $^{\circ}$C under a 16-h photoperiod with a light intensity of 3000 lux and relative humidity of 65-70%. Subculturing was done after every 10-15 days. After 3-4 weeks of subculturing first shooting is observed in callus.

Regeneration of Roots and Development of Complete Plantlets

For root initiation, regenerated shoots (2.5-4.0 cm long) (Figure 1e) were cultured on half strength basal media (MS, B5 and N_6) supplemented with 2% (w/v) sucrose, 0.8% difcobactoagar and different concentrations of NAA and kinetin for 1-2 weeks. The shoots were also tested on growth regulator free half strength basal media. The complete rooted plantlets were washed free of agar and dipped into 0.2% bavistin for 5-10 min, and were then transplanted in small plastic pots containing the mixture of sterile soil, sand and activated charcoal (1:1:1) for hardening (Figure 1f). They were initially acclimatized in a moist chamber at 85% relative humidity and 25 ± 3 °C temperature having provision for aeration at a regular interval. After 3 weeks of hardening the plants were again transferred to 15 cm earthen pots (one

plant per pot) containing the same mixture under semi-shady condition for final hardening. The hardened potted plants were maintained in the open air net house for normal growth under natural condition.

Statistical Analysis

The data presented here are means ± standard error (SE) of at least three replicates. Multiple comparisons of means were performed by ANOVA using statistical software SPSS v. 10 (SPSS Inc., USA). A probability of $P < 0.05$ was considered as significant.

RESULTS

Callus Induction

Among the three basal media used, MS basal medium was found the most effective for callusing from leaf explants (Figure 1a). The explants cultured on MS basal medium supplemented with different concentrations of NAA, kinetin and GA showed significantly varied response for callusing (Table1). The highest callus induction percentage (98%) was obtained with 2 mgl^{-1} NAA, while kinetin induces a maximum of 51.2% callusing on MS medium at 1 mgl^{-1} concentration. GA was found to be least effective for callusing (23.5%) (Table1).On the other hand, on B5 medium callus induction percentage ranged 23.2-79.0%, with the lowest in 3 mgl^{-1} GA and the highest in 3 mgl^{-1} NAA. N_6 medium was the least effective, showing only 14.2-24.0% callusing (Table1).

Table 1: Effects of different PGRs on callus induction from leaf explants of *Adenium multiflorum* Klotzsch in three different basal media. All data are means ± SE of three independent replicates. [a]Data were taken during 1st subculturing. *means differed significantly (ANOVA) at $p < 0.05$

PGRs	Days to Callus Initiation (Mean ± SE)			Callus Induction (%) (Mean ± SE)[a]		
(mg l^{-1})	MS	B5	N_6	MS	B5	N_6
NAA -						
1	28.1 ± 4.3	41 ± 8.5	-	89.0 ± 10.2	66.2 ± 9.3	-
2	20.0 ± 3.7	32 ± 4.8	49 ± 8.7	98.0 ± 12.6	73.4 ± 9.7	24.0 ± 4.1
3	23.0 ± 3.9	27 ± 4.2	47 ± 8.6	97.2 ± 11.8	79.0 ± 9.4	18.2 ± 3.1
Kinetin-						
1	37.0 ± 5.1	53 ± 8.8	-	51.2 ± 08.4	42.1 ± 8.7	-
2	32.0 ± 4.7	47 ± 8.5	-	34.0 ± 04.9	26.3 ± 4.1	-
3	29.0 ± 4.5	38 ± 5.2	51 ± 8.6	32.0 ± 04.6	24.2 ± 4.2	14.2 ± 2.9
GA-						
1	-	-	-	-	-	-
2	52.0 ± 8.9	-	-	22.2 ± 03.9	-	-
3	48.0 ± 8.5	53 ± 9.2	-	23.5 ± 04.1	23.2 ± 3.8	-
*F-Test (Basal Media)**	*F-Test (Basal Media)**		*F-Test (PGRs)**	*F-Test (PGRs)**		

Days to initiate callusing and subsequent growth rate during subculturing were also varied considerably in different basal media and with different PGRs (Table1). In MS medium, callusing was observed a minimum of 20 days after culture initiation with 2 mgl^{-1} NAA to a maximum of 52 days after culture initiation with 2 mgl^{-1}GA. In Gamborg' B5 medium, it varied from 27 to 53 days. Much delayed callus induction, however, was observed on N_6 medium (Table1).

Satisfactory growth rate of calli [expressed in % increased of fresh mass (FM) of calli] was achieved in MS medium with 2 mgl^{-1} NAA (64%) during 3rd subculturing (Figure 2a). Gradual increase in FM of calli during successive subculturing was also obtained in B5 medium with different PGR concentrations

(Figure 2b). The highest growth rate of calli in N_6 medium was only 23%, achieved with 3 mgl^{-1} kinetin during 3rd subculturing regime (Figure 2c).

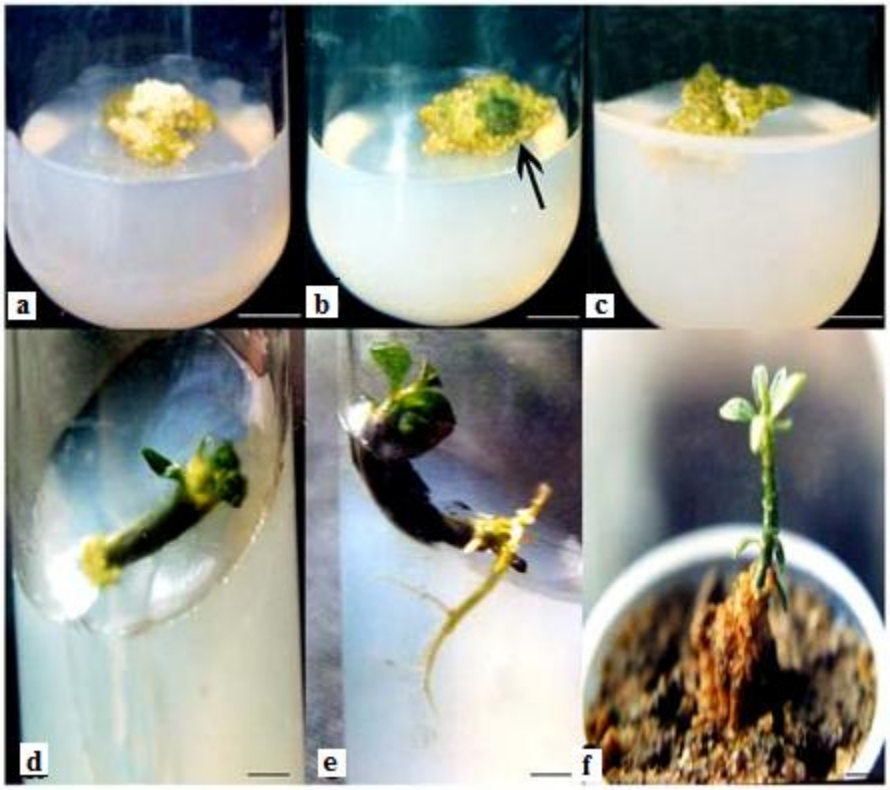

Figure 1: Regeneration of *A. multiflorum* Klotzsch. A - Callus grown form leaf explants. **B -** Shoot bud initiation (→). **C** - Shoot bud differentiation. **D** - Shoot regeneration. **E** - *In vitro* rooting of regenerated shoot. **F** - *Ex vitro* hardening of young regenerated plantlet. *Bars* equal to 0.5 cm in all figures.

Shoot Organogenesis

Effects of different PGRs on shoot induction of *A. multiflorum* were presented in Table 2. NAA of 1 mgl^{-1} and 2 mgl^{-1} were used in combination with different concentrations of kinetin (0.5, 1.0 and 1.5 mgl^{-1}) in three different basal media. In MS medium, days required to shoot organogenesis (Figure 1b-d) after inoculating into shoot induction medium ranged from 27 (in 2.0 mgl^{-1} NAA: 1.5mgl^{-1}kinetin) to 35 (in 1.0 mgl^{-1} NAA: 1.0 mgl^{-1} kinetin). In B5, it ranged between 31 days and 38 days. Much delayed shoot regeneration was occurred in N_6 medium with minimum requirement of 51.2 days (in 1.0 mgl^{-1} NAA: 1.5 mgl^{-1}kinetin). In MS medium, 2.0 mgl^{-1} NAA in combination with 1.5 mgl^{-1} kinetin was found to be optimum for shoot induction (53.9%) and highest number of shoot bud production per responded callus (Table 2). In B5 medium, both 1.0 mgl^{-1} and 2.0 mgl^{-1} NAA were found to be more or less equally effective for inducing shoot, though highest percentage of shoot induction (42.1%) and highest number of shoot bud produced per responded callus (4.2) were lesser in B5 than in MS medium. However, in N_6 medium with all concentrations of NAA and kinetin, shoot induction were very poor (7.3-18.2%), showing very low (0.9-2.1) number of shoot bud per responded callus (Table2).

Root Induction

Effects of different growth regulators on root regeneration were presented in Table 3. Days required to root initiation ranged from a minimum of 10.5 days in MS medium (0.5 mgl^{-1} NAA: 2.5 mgl^{-1}kinetin) to a maximum of 43.2 days in N_6 medium (1.0 mgl^{-1} NAA: 2.0 mgl^{-1}kinetin). Among all combinations, 0.5 mgl^{-1} NAA: 2.5 mgl^{-1} kinetin was found to be optimal for root induction (51.2%), producing highest number of roots/shoot (13.8) in MS medium (Figure 1e). In B5 medium, 3.0 mgl^{-1} kinetin along with 0.5

and 1.0 mgl^{-1} NAA yielded optimum root induction (28.7-32.5%) exhibiting highest number of roots/shoot (7.3-7.6).

Table 2: Effects of different PGRs on shoot induction from callus of *Adenium multiflorum* Klotzsch in three different basal media. All data are means ± SE of three independent replicates. [a,b] Data were taken after 20 days of 1st shoot bud formation.*Means differed significantly (ANOVA) at $p < 0.05$

PGRs	Days to Shoot Regeneration (Mean ± SE)			% of Callus Producing Shoot Bud (Mean ± SE)[a]			Number of Shoot Bud/Responded Callus		
(mg l^{-1})	MS	B5	N_6	MS	B5	N_6	MS	B5	N_6
NAA 1+ Kinetin									
0.5	32.0±4.2	38.0±4.9	59.0±6.2	38.2±4.5	38.7±4.5	7.3±0.85	5.2±0.65	2.4±0.18	1.3±0.22
1.0	35.0±4.7	34.0±4.4	57.0±5.9	41.3±5.1	34.2±4.2	12.8±1.9	6.7±0.71	3.3±0.41	2.1±0.29
1.5	34.0±4.4	36.2±4.5	51.2±6.1	48.1±5.5	33.4±4.1	15.9±2.2	6.9±0.74	4.2±0.52	1.2±0.21
NAA 2+ Kinetin									
0.5	31.0±4.1	34.0±4.4	63.2±6.9	48.1±5.4	37.3±4.4	9.2±1.1	6.2±0.69	2.7±0.31	0.9±0.08
1.0	28.0±3.4	31.0±4.1	59.2±5.6	53.3±5.8	42.1±5.0	13.3±1.8	6.3±0.70	4.1±0.50	1.2±0.14
1.5	27.0±3.2	33.5±4.2	55.7±6.1	53.9±5.9	36.7±4.5	18.2±2.5	9.4±0.98	2.5±0.16	2.0±0.25
*F-Test (Basal Media)**		*F-Test (Basal Media)**		*F-Test (PGRs)**		*F-Test (PGRs)**			

Like callus and shoot induction, N_6 medium was the least effective in inducing root (3.2-13.2%) than the other two basal media used. However, root inducing potentiality of all three basal media in growth regulator free condition was found to be either minimal or negligible.

DISCUSSION

For the first time, a successful *in vitro* regeneration (organogenesis) protocol has been developed in *Adenium multiflorum* Klotzsch. In general, three phases can be distinguished in this procedure. The first is dedifferentiation in which the tissue becomes competent to respond to the organogenic stimulus. It is generally initiated by culturing on an auxin-rich callus-inducing medium and may involve a period of callus growth. The second is induction in which cells become determined to form either a root or a shoot. The explants are cultured on a shoot-inducing medium or root-inducing medium that contains a specific auxin/cytokinin ratio combination. The third is the realization in which the explants grow to an organ (De Klerk *et al.,* 1997; Ochatt *et al.,* 2010).

Selection of suitable explants is very crucial for successful regeneration. Depending on the plant species, only a limited number of cells in an explants show the organogenic response (De Klerk *et al.,* 1997). Therefore, different explants from hypocotyls, leaves and roots showed varied response for callus induction (Hendrawati *et al.,* 2011; Banerjee *et al*., 2012). In the present experiment, juvenile leaf explants of smaller size were utilized to initiate callusing due to the fact that smaller size of explants provide less chance of contamination, as well as larger leaves showed total loss of morphogenic/organogenic potentiality (Mujib 1997). Barring N_6 medium, initiation of calli from leaf explants did not pose a major problem in the present material. In N_6 medium (supplemented with NAA, kinetin or GA, the leaf segments remain green for long period with very slow process of callus induction/growth. MS basal medium showed varied responses on callusing when supplemented with

NAA, kinetin or GA, and was the most effective for callusing on leaf explants as also supported by Sehrawat *et al*. (2001).

Table 3: Effects of different PGRs on root induction of *Adenium multiflorum* Klotzsch in three different basal media. All data are means ± SE of three independent replicates. [a,b]Data were taken after 10 days of 1st rooting. *Means differed significantly (ANOVA) at $p < 0.05$

PGRs	Days to Root Initiation (Mean ± SE)			Root Induction % (Mean ± SE)[a]			Number of Roots/Shoot (Mean ± SE)[b]		
(mg l^{-1})	MS	B5	N_6	MS	B5	N_6	MS	B5	N_6
NAA 0.5 + Kinetin									
2.0	15.3±1.9	28.0±3.9	39.0±4.2	12.5±2.0	13.2±2.2	3.2±0.45	4.2±0.55	3.1±0.21	1.5±0.22
2.5	10.8±1.4	24.0±3.4	37.0±3.9	51.2±6.1	24.5±3.2	9.5±1.1	13.8±2.4	7.2±0.81	2.1±0.29
3.0	11.5±1.2	26.2±3.5	31.2±4.1	44.5±5.5	28.7±3.6	13.2±2.2	10.2±1.4	7.6±0.83	2.7±0.31
NAA 1.0+ Kinetin									
2.0	21.0±3.1	24.0±3.4	43.2±4.9	25.2±3.4	11.1±2.1	4.1±0.52	5.0±0.59	2.4±0.31	2.1±0.28
2.5	18.0±2.4	21.0±3.1	39.2±3.6	37.5±4.3	23.2±3.1	7.3±0.81	8.3±0.70	5.2±0.50	2.8±0.31
3.0	11.2±2.2	23.5±3.2	35.7±4.1	46.4±5.2	32.5±4.2	9.7±1.2	11.4±1.5	7.3±0.86	3.2±0.41
*F-Test (Basal Media)**				*F-Test (Basal Media)**			*F-Test (PGRs)**		*F-Test (PGRs)**

The formation of an unorganized mass of plant cells (callus) in medium is definitely controlled by growth regulatory substances (auxins, cytokinins) present in the medium (Shah *et al.,* 2003). The specific concentration of plant regulators needed to induce callus, varies from species to species, medium to medium and even depends on the source of explants (Charriere *et al.,* 1999). In *A. multiflorum***,** satisfactory callus inducing percentage (89-98%) was obtained in MS medium fortified with auxins like NAA, and slightly lesser response was achieved in B5 medium (66-79%). In many cases, it has been observed that 2, 4-D is the choice of auxins for callus induction and subculturing of grasses (Bhaskaran and Smith 1990; Chaudhury and Qu 2000). The present study further demonstrates that in the media supplemented with low concentration of kinetin (1.0 mgl^{-1}) the callus induction was more significant (51.2% in MS medium and 42.1% in B5 medium) than with higher concentrations like 2.0 and 3.0 mgl^{-1} of kinetin. This is noted for the first time in *Adenium* and remains in accordance with previous experimental results on rye grasses and tall fescue (Alpeter and Posselty 2000; Bai and Qu 2001; Bradely *et al.,* 2001), indicating that the addition of a low concentration of cytokinin in callus culture medium often enhances callus regeneration. Subsequent growth of callus in media, expressed in percent FM increased, was more rapid in the present material with regular subculturing of callus, which probably excludes the undesirable phenolic or other compounds released by plant tissues to the medium reaching inhibitory concentrations (Pan and Van Staden 1998).

Assessments of PGRs in the current experiment showed that NAA as an auxins and kinetin as a cytokinin was beneficial in both MS and B5 basal media for optimum shoot induction of *A. multiflorum*. The highest percentage of callus producing shoot buds was 53.9% on MS medium supplemented with 2.0 mgl^{-1} NAA: 1.5 mgl^{-1} kinetin. High frequency shoot bud formation and plant regeneration were also achieved in *Dianthus chinensis*, when culture medium contained both cytokinin and auxin (Kantia and Kothari 2002). In *A. obesum*, however, the presence of BA (as cytokinin) alone in the medium appeared to be optimal for shoot induction (Kanchanpoom *et al*., 2010). This suggests that bud formation in Adeniums may require cytokinin. A combination of cytokinin and auxin did not evoke a better response in *A.*

obesum than cytokinin alone. This is probably due to the differences in endogenous levels of growth regulators or to a difference in sensitivity to growth substances (Trewawas and Cleland 1983). The present experimental results are in concurrence with the view that there are differences in the organogenetic potential between plant families, genera, species and even different genotypes of a species (George 1996). In the present material, there was a considerable increase in shoot bud number on higher kinetin level (1.5 mgl^{-1}). The leaves were healthy with green colour and did not show any sign of vitrification (Figure 1d-f). Thus, it can be inferred from the above results that shoot induction of *A. multiflorum* was easily achieved for all NAA and kinetin treatments in both MS and B5 basal media, though higher kinetin levels were considered as optimal for *in vitro* regeneration of shoots in this endangered ornamental plant.

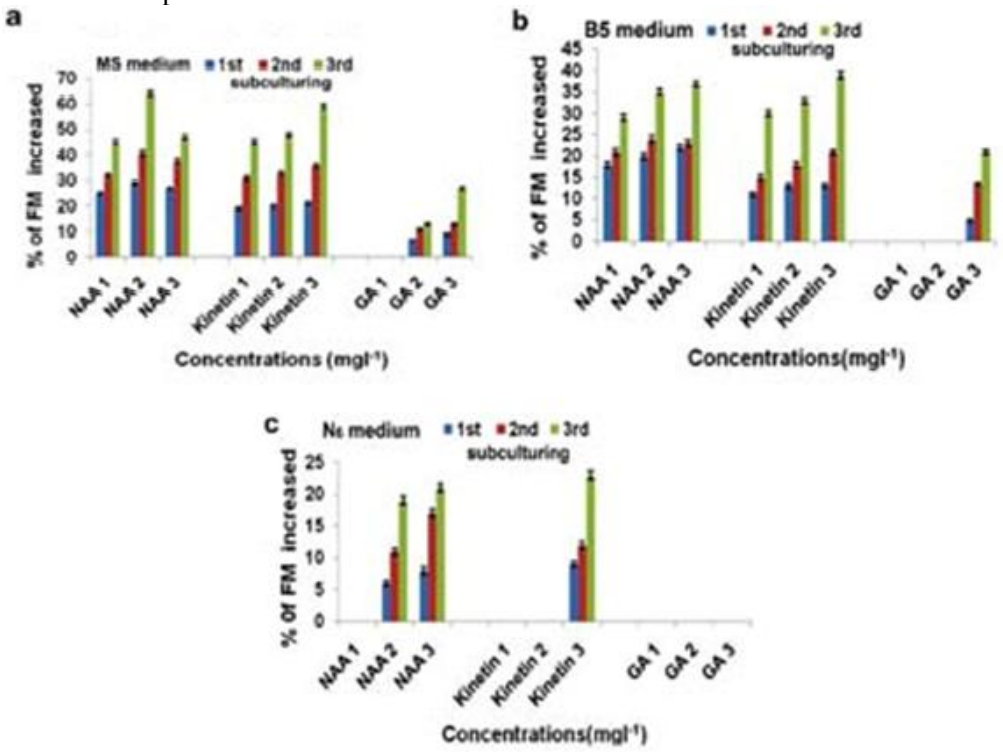

Figure 2: Fresh Mass (FM) increase percentage of calli during 1st , 2nd and 3rd subculturing on different media supplemented with three different PGRs-NAA, Kinetin and GA at concentrations of 1 mgl^{-1}, 2 mgl^{-1} and 3 mgl^{-1}. A - on MS medium. **B** - on B5 medium. **C** - on N_6 medium.

Induction of roots is thought to be pivotal to form the complete plantlets. Regenerated shoots obtained from the shoot induction media did not produce roots in the same medium (full strength). However, when 2.5-4.0 cm elongated shoots were placed on half strength MS and B5 basal media supplemented with NAA and kinetin, roots were induced within 10-12 days (Figure 1e). In several woody plants, rooting was facilitated by a reduced (half or quarter) concentration of salts in the culture medium (Rout *et al*., 2000), though in *Gomortega keule* (Chilean tree) there was no such advantage of a reduced concentration of salts in rooting (Munoz-concha and Davey 2011). The highest rooting in the present material was observed in 0.5mgl^{-1} NAA: 2.5 mgl^{-1} kinetin for MS medium and in 1.0 mgl^{-1} NAA: 3.0 mgl^{-1} kinetin for B5 medium. The result revealed that *A. multiflorum* required the presence of both auxin and cytokinin in the medium for root induction, and thus distinctly differed from *A. obesum* which did not require the presence of auxins in the medium for root induction (Kanchanpoom *et al*., 2010).

Completely rooted plants were hardened initially in a moist chamber and finally in an open air net house under normal condition (Figure 1f). Inclusion of activated charcoal in the potting mixture facilitated

further rooting of regenerated *A. multiflorum* plants during hardening. Similar results were observed in many ornamental pot plants (Rout *et al*., 2000), *Cinnamomum camphera* (Babu *et al*., 2003) etc., and mentioned that activated charcoal may adsorbs growth regulators and waste exudates released by plants or create partial darkness which is similar to the underground environment, thus causes increased rooting. The survival of plantlets was 70-72% under natural photoperiod. The *A. multiflorum* plantlets thus obtained were phenotypically normal looking during subsequent vegetative development.

In conclusion, this is the first report on the regeneration of *A. multiflorum* plants *in vitro*. The micropropagation protocol developed here is also highly relevant since propagation by seeds is restricted in this plant. Finally, seed propagation does not maintain genetic identity/fidelity, which is of paramount importance. Therefore, regeneration via callus culture will provide a basis for genetic improvement of Adeniums by conventional breeding and somatic cell technologies. Further optimization is needed to increase the efficiency and frequency of regeneration and to reduce the total regeneration period.

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