Middle East Journal of Agriculture Research
ISSN 2077-4605
Volume : 07 | Issue : 02 | April-June | 2018
Pages: 569-582

Micropropagation and conservation of *Odontonema cuspidatum* plant by tissue culture

Salwa S. Sakr[1], Mohamed A. El-Khateeb[1], M.A. El-Shamy[2] and Asmaa E. Abd Elhafez[2]

[1]Department of Ornamental Horticulture, Faculty of Agriculture, Cairo University, Giza, Egypt
[2]Department of Botanical Garden Research, Horticulture Research Institute, Agriculture Research Center, Giza, Egypt

Received: 05 Mar. 2018 / Accepted: 20 May 2018 / Publication date: 28 May 2018

ABSTRACT

This study was carried out to reach a well defined protocol for *in vitro* propagation of *Odontonema cuspidate* plant and conservation for germplasm storage. The obtained results could be summarized as follows: Using NaOCl at 2% for 25 min was the most effective concentration for sterilizing the shoot tips of. The survival was 67% while contamination was 33%. Culturing of the explants on full MS medium supplemented with 0.5 mg/l TDZ was positively effective on number of shoots. Supplementation of the culture medium with 1mg/l IBA and 2 mg/l NAA resulted in the highest number of roots. The rooted plantlets were cultivated in pots containing peat moss+ sand at the ratio of 3:1 (v/v) and covered with plastic sheets for three weeks. After removal of the sheets, the seedlings were left for additional three weeks under the greenhouse conditions. Shoot tips from *In Vitro* shoot cultures derived from multiplied shoots were collected and suspended in sodium alginate (5 %) and dropped into 1.1 g/100ml calcium chloride ($CaCl_2.2H_2O$) then cultured on half-strength MS medium without sucrose for synthetic seeds production. Concerning addition of two layers of capsules coat, the inhibition percent of shoot tips which were exposed to calcium chloride solution for 15 min in the first layer was the most suitable.

Key words: Micropropagation, In vitro, Tissue culture, *Odontonema cuspidatum*, conservation, encapsulation, Synthetic seeds.

Introduction

The genus *Odontonema* belongs to the Acanthaceae Family which is composed of about 346 genera with almost 4300 species, widely distributed in tropical regions of the world but are poorly represented in temperate regions. Nodal explants collected from mature plants of *Andrographis lineata* were treated with 0.1% $HgCl_2$ (w/v) for 2 min before culturing them vertically on agar gelled MS medium (Mohammed *et al.,* 2016). The internode explants of *Asteracantha longifolia* Nees were used for high frequency plant regeneration on Murashige and Skoog (MS) medium supplemented with NAA, BA at different concentrations (Kumar and Nandi, 2015). Shoots of *Ruellia tuberosa* were transferred to MS medium supplemented with 1.0 mg/l of NAA and 1.0 mg/l of IBA at half strength of MS medium was found to be the most suitable for root induction (Lakshmanan and Gabriel, 2015). The rooted plants of *Rhinacanthus nasutus* (L.) Kurz were successfully transplanted in plastic cups (6 cm diameter) containing (1:1) garden soil and sand (Cheruvathur and Thomas, 2014).

For encapsulation, 4- to 5-mm-long nodal segments were isolated after 5 weeks from subculturing. Nodal segments were mixed with a liquid modified MS medium supplemented with 3% Na-alginate and hardened in 75 mM $CaCl_2$. For sowing in non-sterile conditions, a second layer containing water, MS medium, 0.2 M mannitol, or 0.5 g/l activated charcoal, was added to the beads (Pinker and Abdel-Rahman, 2005). Shoot buds derived from calli of *Pogonatherum paniceum* were used for encapsulation in liquid MS medium supplemented with 3% sucrose and two different alginate matrices with a 20-min dipped in 2% $CaCl_2$ and 0.3% bavistin (w/v). The capsule with 3.0% sodium alginate (w/v) and 1% activated carbon (w/v) showed a higher conversion rate (61.58%) and stronger plantlets under non-aseptic conditions (Wang *et al,* 2007). Synthetic seeds of *Gerbera jamesonii* were formed when the micro shoots and somatic embryos were encapsulated using 3.0%

Corresponding Author: Asmaa E. Abd Elhafez, Department of Botanical Garden Research, Horticulture Research Institute, Agriculture Research Center, Giza, Egypt.
E-mail address: soma.8573@yahoo.com

sodium alginate solution and beads were solidified using 100 mM calcium chloride ($CaCl_2.2H_2O$) solution. High germination rate (75-95%) was achieved after one to three months storage, whereas low germination rate (8-50%) was obtained after four to six months storage (Taha *et al*, 2009). The encapsulation of *Decalepis* nodal segments was significantly affected by the concentrations of sodium alginate (Na-alginate) and calcium chloride ($CaCl_2.2H_2O$). A gelling matrix of 4 % Na-alginate and 100 mM $CaCl_2.2H_2O$ was found most suitable for the production of ideal Ca-alginate beads. (Sharma and Shahzad, 2012). The seven weeks old protocorm like bodies (PLBs) of *Rhynchostylis retusa* were encapsulated with 2 to 3% sodium alginate (w/v) in the BM medium and exposed to 50 to 100 mM calcium chloride solution ($CaCl_2.2H_2O$). It was found that 3% sodium alginate dipped in 100mM $CaCl_2.2H_2O$ solution was incubated for 40 min in orbital shaker to produce firm, transparent and uniform beads. The synthetic seeds were stored at 4°C and 25°C (Sharma, 2014). *In vitro* grown shoot tips and nodal segments in *Rhinacanthus nasutus* were encapsulated at different concentrations of sodium alginate (2- 5 % w/v) and calcium chloride (25 to 200 mM). Maximum shooting regrowth response (95%) of encapsulated shoot tips produced multiple shoots (6.0 ± 1.8) and re-growth was observed on MS medium supplemented with 6- benzylaminopurine BAP (2.0 mg/l) and nodal segments re-growth response (85%) was observed with of multiple shoots (3.5 ± 0.5) cultured on MS medium with 2.0 mg/l BAP (Elangomathavan *et al*, 2017a). The perfect synthetic seeds were obtained through a combination of 4% sodium alginate and 100 mM calcium chloride in half strength MS medium with 3% sucrose. The maximum shoot re-growth response of encapsulated nodes (95%) and shoot tips (90%) were obtained in MS medium supplemented with 1.0 mg/l BAP after 6 weeks of culture (Elangomathavan *et al,* 2017b).

Therefore, this study is to investigate the most *in vitro* conservation system technique which can be used in conjunction with micropropagation for *Odontonema cuspidatum*. It can be used for germplasm storage or as a mean to reduce the need for transferring and subculturing during off-season periods that could be useful in a large-scale.

Material and Methods

The experiments were carried out in the Laboratory of Tissue Culture, Zohria Botanical Garden, Cairo, Horticulture Research Institute, Agriculture Research Center, Ministry of Agriculture, during the period from 2014 to 2017. The aim of this study was to develop a method of explants encapsulation which to be used for storage of the explants.

Plant material:

The shoot tips were used as explants from mother plants growing at the open field condition in Zohria Botanical Garden.

Fig. 1: *Odontonema cuspidatum*

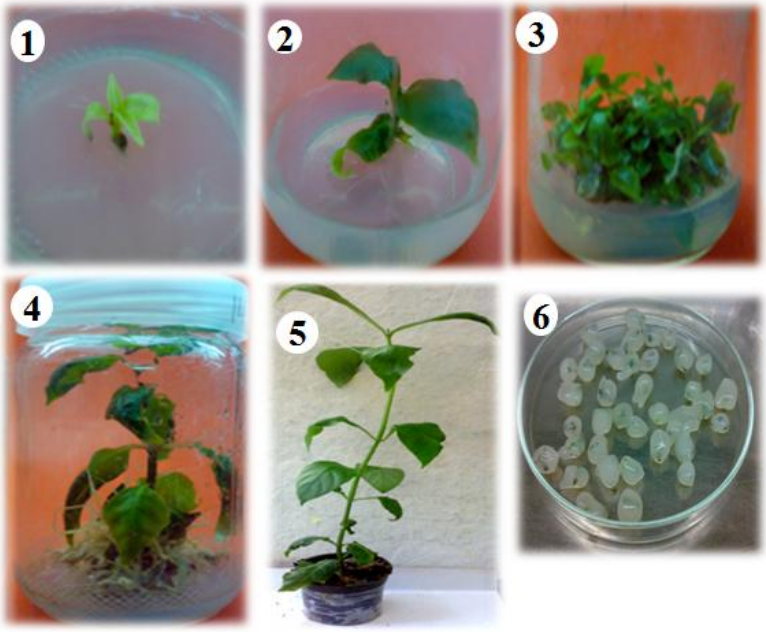

Fig. 2: Micropropagation and conservation of *Odontonema cuspidatum*

1-Surface Sterilization 2- Establishment Stage 3-Multiplication Stage
4- Rooting Stage 5- Acclimatization Stage 6- Artificial seeds.

Culture room condition:

Cultures of *Odontonema cuspidatum* were placed in a growth chamber under 28±2°C and 16-h photoperiod. The light was provided with white fluorescent light lamps of 2000 lux.

Experimental design and statistical analysis:

A factorial experiment in a complete randomize design was employed in all experiments. Analysis of variance was used to show statistical differences between treatments using L.S.D at 5% probability level (Snedecor and Cochran, 1989).

Culture media:

The Murashige and Skoog (MS) medium was used for culturing the explants of *Odontonema cuspidatum*. Media were solidified and supplemented with 7.0 g/l agar. Sucrose at 30.0 g/l was added as a source of carbohydrate. The pH was adjusted to 5.7. Twenty ml medium were poured in 150 ml jars and sterilized by autoclaving under steam pressure at 1.5 bar at 121°C for 20 min. Each treatment consisted of 3 jars. In each jar, three shoots were cultured separately.

Experimental treatments

Surface sterilization of explants:

The explants were excised from the mother plants and then washed by soapy water for 10 min. The explants were rinsed under a running tap water for 1 hour. The explants were then sterilized by immersion in a sodium hypoclorite solution at the rate of 0.5, 1.0, 1.5, 2.0 and 2.5 % and mercuric chloride ($HgCl_2$) at the rate of 0.0, 0.5, 1.0 and 2.0 mg/l plus 2-3 drops of Tween -20 for 25 min. After sterilization treatment the explants were then rinsed 5 times in a sterilized distilled water to remove all traces of the disinfectant. All steps of the sterilization method had been done under aseptic condition

inside the culture cabinet (Laminar air flow) using sterilized instruments. Twenty treatments were initiated from the use of explants.

One drop of Tween -20 (polyoxyethlene sorbitan monolaurate) was used as a wetting agent per 100 ml of sterilizing solution for each treatment. Each treatment consisted of three jars. In each jar, three explants were sterilized. At the end of the experiments, the obtained data included: survival percentage, contamination percentage and mortality percentage.

Establishment stage:

In this part, the explants were used to study the effect of different treatments on establishment stage. Eighteen treatments were initiated from the use of explants.

The media employed at this stage were MS at full, half and quarter strength (4.4, 2.2 and 1.1 g/l). It was supplemented with Indole -3- butyric acid (IBA) at 0.0, 0.1, 0.5, 1.0, 2.0 and 3.0 mg/l to initiate the shoots. Each treatment consisted of three jars. The recorded data were as follows: shoot length (cm) and number of leaves.

Multiplication stage:

In these experiments explants were cultured for multiplication stage. Multiplication media consisted of MS basal nutrient medium supplemented with thidiazuron (TDZ) at 0.0, 0.01, 0.1, 0.5, 1.0 or 2.0 and naphthalene acetic acid (NAA) at 0.0, 0.1, 0.5 or 1.0 mg/l. The different concentrations of TDZ and NAA were also used either separately or in combination. The recorded data were as follows: shoot length (cm), number of leaves and number of shoots.

Rooting stage:

This experiment was carried out to study the effect of culture medium supplemented with IBA at 0.0, 0.5, 1.0, 2.0 or 4.0 mg/l and NAA at 0.0, 0.5, 1.0, 2.0 or 4.0 mg/l. In combinations between them to study their effect on growth and root formation of *Odontonema cuspidatum* shoots. Three shoots at the length of 3.0 cm, resulted from the multiplication stage were cultured in each jar (350 ml), which contained 50 ml of rooting medium. After 45 days on the rooting media the following data were recorded: number of roots and root length (cm).

Acclimatization stage:

Rooted plantlets were pricked out singly into 10 cm plastic pots filled with a mixture of peatmoss at 1.0, 2.0 or 3.0 and sand at 0.0, 1.0, 2.0 or 3.0 (v/v). To maintain cultures at high humidity, the pots were covered with clear transparent plastic sheets for three weeks. The plastic covers were then gradually removed to reduce humidity and adapt plantlets to greenhouse conditions. The recorded data were as follows: plantlet length (cm) and number of leaves.

Encapsulation for conservation:

a. Preparation of sodium alginate.

Sodium alginate was added in three concentrations i.e., 3.0, 4.0 and 5.0 % (w/v) to full or half MS liquid medium with 3.0 % (w/v) sucrose or without sucrose then sterilized into autoclave for 20 min under steam pressure of 1.5 bar at 121°C and allowed to cool for one hour.

b. Preparation of calcium chloride solution.

Calcium chloride ($CaCl_2.2H_2O$) was dissolved at 1.1, 2.2, 3.3 g/100ml as in a distilled water and then sterilized in autoclave for 20 min under steam pressure of 1.5 bar at 121°C and allowed to cool under room temperature.

c. Explants encapsulation.

Encapsulation was accomplished by micro cuttings grown culture in sodium alginate solution and then they were dropped into calcium chloride solution for 30 min to produce calcium alginate capsules. These capsules were placed on sterilized filter paper and dehydrated by air drying in a Laminar flow chamber at room temperature for 30 min for the first experiment. Solution was prepared to produce clear, uniformal capsules within ion exchange duration of 30 min. The obtained data were recorded as follows: mortality (%), inhibition (%) and sprouting (%).

In the second experiment of encapsulation, the explants were dropped into sodium alginate solution and exposed to calcium chloride solution for 5, 10 and 15 min, followed by dropping them into sodium alginate, which was followed by calcium chloride for 30 min and them left to dehydrate in the second layer. Addition of two layers from calcium alginate was to improve the encapsulated explants regrowth after storage periods. The recorded data were as follows: inhibition (%) and sprouting (%).

d. Capsules storage.

Encapsulated cultures of each treatment were collected after 30 min and incubated at 28ºC day and night temperature and stored in jars containing half MS medium with 3.0% (w/v) sucrose or without sucrose and incubated at 16-h photoperiod. Each treatment consisted of three replicates, which contained five capsules. The data were collected for each treatment after 2, 4 and 8 weeks of storage.

Results and Discussion

Effect of different concentrations of NaOCl and $HgCl_2$ on survival, mortality and contamination (%) as surface sterilization of *Odontonema cuspidatum*.

Results recorded in Table (1) show that NaOCl at 1% gave the highest survival value of explants (22%) as compared with the other NaOCl concentrations. However, the percentage of mortality (44.5%) and the percentage of contaminated explants (33.5%) were recorded as a result of immersing the explants in 1% NaOCl (%). On the other hand, data indicated that increasing the concentration of NaOCl (%) and $HgCl_2$ (mg/l) as immersion for 25 min decreased the percentage of explants survival and the contaminated explants, while the mortality percentage of explants was increased. The data of the interaction between the concentration of NaOCl and $HgCl_2$ indicated that the best percentage of explants survived (67%), the percentage of contaminated explants (33%) and the mortality percentage (0.00%) were obtained when the explants were immersed in 2% NaOCl without $HgCl_2$ for 25 min. This result shows that the presence of $HgCl_2$ at any concentration was depressing as well decreased the survival % of the explants. The obtained results were in harmony with those obtained on *Clinacanthus nutans* by **Gunasekaran (2014).**

Effect of MS medium strength and IBA concentrations on establishment stage of *Odontonema cuspidatum*:

For the establishment stage, data in Table (2) showed that the different concentrations of IBA (0.0, 0.1, 0.5, 1.0, 2.0 or 3.0 mg/l) and MS-strength (full, half or quarter) had a significant effect on shoot length (cm) and number of leaves.

Shoot length (cm):

Using MS-medium at full strength produced the longest shoot (1.35 cm), while the explants cultured on quarter-strength of MS medium gave the shortest shoot (0.81 cm). The longest shoot (2.00 cm) was obtained on full-strength MS medium supplemented with 2.0 mg/l IBA.

Table 1: Effect of NaOCl concentration (%) and $HgCl_2$ concentration (mg/l) for 25 min on percentages of survival, mortality and contamination at surface sterilization of *Odontonema cuspidate*

	Survival (%)					**Mortality (%)**					**Contamination (%)**				
$HgCl_2$ (mg/l) / NaOCl (%)	**0.0**	**0.5**	**1.0**	**2.0**	**Mean (A)**	**0.0**	**0.5**	**1.0**	**2.0**	**Mean (A)**	**0.0**	**0.5**	**1.0**	**2.0**	**Mean (A)**
0.5	33	22	23	11	**22.3**	0.0	22	33	78	**33.3**	67	56	44	11	**44.5**
1	44	22	22	0.0	**22**	0.0	22	56	100	**44.5**	56	56	22	0.0	**33.5**
1.5	44	33	11	0.0	**22**	0.0	44	67	100	**52.8**	56	23	22	0.0	**25.3**
2	67	22	0.0	0.0	**22.3**	0.0	56	78	100	**58.5**	33	22	22	0.0	**19.3**
2.5	33	11	0.0	0.0	**11**	23	67	100	100	**72.5**	44	22	0.0	0.0	**16.5**
Mean (B)	**44.2**	**22**	**11.2**	**2.2**		**4.6**	**42.2**	**66.8**	**95.6**		**51.2**	**35.8**	**22**	**2.2**	
$LSD_{0.05}$ for															
	NaOCl(A)				**13**			**14**					**14**		
	$HgCl_2$ (B)				**12**			**13**					**12**		
	(AxB)				**26**			**27**					**28**		

Table 2: Effect of MS medium strength supplemented with different concentrations of IBA on shoot length (cm) and number of leaves at establishment stage of *Odontonema cuspidatum*

	Shoot length (cm)							No. of leaves						
IBA (mg/l) / MS (g/l)	**0.0**	**0.1**	**0.5**	**1.0**	**2.0**	**3.0**	**Mean (A)**	**0.0**	**0.1**	**0.5**	**1.0**	**2.0**	**3.0**	**Mean (A)**
4.4	1.13	1.13	1.25	1.38	2.00	1.25	**1.35**	5.50	6.25	6.50	6.50	8.75	6.75	**6.71**
2.2	0.88	0.94	0.94	1.00	1.13	0.95	**0.97**	4.25	4.75	5.25	5.50	5.75	5.50	**5.17**
1.1	0.69	0.69	0.81	0.88	0.94	0.88	**0.81**	3.50	4.25	4.75	5.25	5.50	5.25	**4.75**
Mean (B)	**0.90**	**0.92**	**1.00**	**1.08**	**1.35**	**1.02**		**4.42**	**5.08**	**5.50**	**5.75**	**6.67**	**5.83**	
$LSD_{0.05}$ for														
MS (A)				**0.11**							**0.31**			
IBA (B)				**0.15**							**0.44**			
(AxB)				**0.31**							**0.77**			

Number of leaves:

It is clear from data presented in Table (2) that the various treatments significantly affected on the number of leaves. The highest mean value for number of leaves (6.71) was obtained at MS medium full-strength. Medium supplemented with 2.0 mg/l IBA significantly increased number of leaves (6.67). The interaction between at MS medium full-strength and 2.0 mg/l IBA produced the highest value for number of leaves (8.75).

Effect of different concentrations of TDZ and NAA on multiplication stage of *Odontonema cuspidatum*:

Shoots length (cm):

It is clear from data exhibited in Table (3) that the shoot length was decreased due to raising TDZ concentrations up to 0.5 mg/l, giving the longest shoots (4.17cm) then decreased with raising its concentration. The cultured shoots on MS medium containing NAA at 1.0 mg/l gave the shortest shoot (1.86 cm). The interaction between TDZ and NAA concentrations resulted in the longest shoot (5.33 cm) was recorded on the medium supplemented with 0.5 mg/l TDZ.

Number of leaves:

The number of leaves as shown in Table (3) revealed a similar trend as that of the shoots length obtained as a result of TDZ and NAA treatments on the number of leaves. The number of leaves was decreased with increasing TDZ concentrations; the highest number of leaves (11.00) was recorded at 0.0 mg/l TDZ. The shoots were cultured on 0.0 mg/l NAA gave the highest number of leaves (9.56). The interaction between TDZ and NAA concentrations showed that the highest number of leaves (12.67) was recorded for control treatment.

Number of shoots:

Concerning the effect of TDZ concentrations, the results illustrated in Table (3) showed that the number of shoots was decreased with raising TDZ concentrations up to 0.5 mg/l, giving the highest mean value for the number of shoots (34.67) at 0.5 mg/l TDZ. Concerning the effect of NAA concentration, the highest mean value for number of shoots was obtained from control treatment (20.78). Regarding the interaction between TDZ and NAA concentrations, it was found that 0.5 mg/l TDZ gave the highest number of shoots (50.33) at 0 mg/l NAA.

These results seemed to be in harmony with those obtained by Karuppusamy and Kalimuthu (2010) on *Andrographis neesiana* cultivated on MS medium containing 10 µM TDZ, recording the highest number of shoots.

Effect of different concentrations of IBA and NAA on rooting stage of *Odontonema cuspidatum:*

Data presented in Table (4) demonstrated that IBA in the presence of NAA levels significantly affected the rooting of *Odontonema cuspidatum* .Concerning IBA concentration, it was found that 1.0 mg/l IBA gave the highest mean value for the number of roots (11.53) and root length (4.70 cm). However, NAA at 2.0 mg/l resulted in the greatest number of roots and root length (12.87 and 4.50 cm, respectively) as compared with the other concentrations. Regarding the interaction between IBA and NAA, it was found that application of 1.0 mg/l IBA and 2.0 mg/l NAA produced the highest number of roots and root length (18.67 and 6.83 cm, respectively).

A similar trend was reported by Hassan *et al*. (2011) who obtained rooted shoots of *Phlogacanthus thyrsiflorus* Nees cultured on half strength MS supplied with 0.5 mg/l IBA + 0.5 mg/l NAA.

Table 3: Effect of different concentrations of TDZ and NAA on shoot length (cm), number of leaves and number of shoot at multiplication stage of *Odontonema cuspidatum* .

TDZ (mg/l) \ NAA (mg/l)	Shoot length (cm)					No. of leaves					No. of shoots				
	0.0	**0.1**	**0.5**	**1.0**	**Mean (A)**	**0.0**	**0.1**	**0.5**	**1.0**	**Mean (A)**	**0.0**	**0.1**	**0.5**	**1.0**	**Mean (A)**
0.00	1.00	1.00	1.00	1.00	**1.00**	12.67	11.33	10.67	9.33	**11.00**	1.00	1.00	1.00	1.00	**1.00**
0.01	1.00	1.00	1.00	1.00	**1.00**	11.33	10.00	9.33	8.67	**9.83**	1.00	1.00	1.00	1.00	**1.00**
0.10	1.00	1.00	1.00	1.00	**1.00**	9.33	8.67	8.00	7.33	**8.33**	24.00	12.67	1.00	1.00	**9.67**
0.50	5.33	4.33	3.83	3.17	**4.17**	8.67	7.33	7.33	6.67	**7.50**	50.33	38.33	34.33	15.67	**34.67**
1.00	4.17	3.67	3.33	2.67	**3.46**	8.00	6.67	5.33	5.33	**6.33**	26.67	22.33	20.33	13.67	**20.75**
2.00	3.17	2.83	2.67	2.33	**2.75**	7.33	6.67	5.33	5.33	**6.17**	21.67	16.33	12.33	6.33	**14.17**
Mean (B)	**2.61**	**2.30**	**2.14**	**1.86**		**9.56**	**8.44**	**7.67**	**7.11**		**20.78**	**15.28**	**11.67**	**6.45**	
LSD $_{0.05}$ for															
		TDZ(A)		0.16				0.88					0.20		
		NAA(B)		0.13				0.72					0.17		
		(AxB)		0.33				1.76					0.41		

Table 4: Effect of different concentrations of IBA and NAA on number of roots and root length (cm) at rooting stage of *Odontonema cuspidatum*

	No. of roots						Roots length (cm)					
NAA (mg/l) / **IBA (mg/l)**	**0.0**	**0.5**	**1.0**	**2.0**	**4.0**	**Mean (A)**	**0.0**	**0.5**	**1.0**	**2.0**	**4.0**	**Mean (A)**
0.00	1.67	5.67	8.33	10.33	9.67	**7.13**	2.17	1.83	1.67	1.50	1.17	**1.67**
0.50	4.33	6.67	9.33	11.67	10.67	**8.53**	3.17	3.83	4.50	3.67	2.67	**3.57**
1.00	6.67	8.67	11.33	18.67	12.33	**11.53**	3.83	4.67	4.83	6.83	3.33	**4.70**
2.00	8.67	8.33	10.67	13.33	11.33	**10.47**	3.67	4.83	5.17	5.83	2.67	**4.43**
4.00	8.33	8.33	9.67	10.33	9.67	**9.20**	3.33	4.17	4.33	4.67	2.67	**3.83**
Mean (B)	**5.93**	**7.47**	**9.87**	**12.87**	**10.73**		**3.23**	**3.87**	**4.10**	**4.50**	**2.50**	
$LSD_{0.05}$ for												
IBA(A)						**0.41**						**0.24**
NAA(B)						**0.41**						**0.24**
(A x B)						**0.93**						**0.54**

Effect of different concentrations of peatmoss and sand at acclimatization stage of *Odontonema cuspidatum*:

Data in Table (5) showed that the plantlets of *Odontonema cuspidatum* were successfully lifted when they were transferred to be cultured in pots containing a (v/v) mixture of peatmoss (at 1, 2, or 3) combined with sand (at 0, 1, 2 or 3) and covered by polythene sheets in the greenhouse. After four weeks, the sheets were removed and left for additional four weeks under plastic house condition. The plantlet length and number of leaves were recorded after 5 weeks.

The results revealed that the highest mean value for the length of the plantlet (11.56 cm) for sand at 1.0 portion without effect of peat moss, however the mean value for peat moss effect was 11.67cm at 3 portions without effect of sand. Moreover, as the volume of sand increased more than 2 portions the length of the plantlets mean value decreased, while peat moss increased the length as its portion was increased.

The longest plantlet (15.33 cm) had been recorded when the plantlets were cultured in a mixture of peatmoss and sand at 3:1(v/v). The highest number of leaves was obtained (17.33 leaves) when the plantlets were cultured in mixture of a peatmoss and sand 3:1(v/v) .These findings are in line with those reviewed by Girija *et al.* (1999) on *Crossandra infundibuliformis* (L.) which were grown successfully in sand and vermiculite (1:1).

Table 5: Effect of different mixtures from peatmoss: sand (v/v) on plantlet length (cm) and number of leaves after two months on acclimatization stage of *Odontonema cuspidatum.*

	Plantlet length (cm)					**No. of leaves**				
Sand / Peatmoss	0.0	1.0	2.0	3.0	**Mean (A)**	0.0	1.0	2.0	3.0	**Mean (A)**
1.0	7.33	8.67	9.67	10.33	**9.00**	8.67	10.67	11.33	10.00	**10.17**
2.0	7.33	10.67	11.33	10.33	**9.92**	8.67	12.00	11.33	10.67	**10.67**
3.0	7.33	15.33	12.33	11.67	**11.67**	8.67	17.33	12.67	11.33	**12.50**
Mean (B)	**7.33**	**11.56**	**11.11**	**10.78**		**8.67**	**13.33**	**11.78**	**10.67**	
$LSD_{0.05}$ for										
Peatmoss (A)					**0.46**					**0.88**
Sand (B)					**0.53**					**1.02**
(A x B)					**0.92**					**1.76**

Effect of different concentrations of sodium alginate and calcium chloride on conservation:

Shoot tips from *in vitro* shoot cultures derived from multiplied shoots were encapsulated in sodium alginate and calcium chloride. Different concentrations of sodium alginate and calcium chloride solutions were tested in order to optimize the size, shape and texture of synthetic seeds for shoot tips. The perfect encapsulated shoot tips were obtained through a highest percentage of inhibition.

Data presented in Table (6) showed that increasing calcium chloride concentrations from 1.1 to 3.3 g/100 ml significantly increased the mortality percentage since, the percent increased from 1.11 to 14.44%. Howerer, the percentages of inhibition and sprouting were decreased by increasing of calcium chloride. Addition of sodium alginate at 3, 4 or 5% gave 0.0, 1.67 and 20 % of mortality and 0.0, 2.78 or 68.89 % of inhibition with significant differences between the treatments after 8 weeks of the conservation. The sprouting percent was significantly decreased by increasing of sodium alginate. The interaction between calcium chloride and sodium alginate gave the highest inhibition for shoot growth (81.67 %) at 1.1 g/100ml calcium chloride and 5% sodium alginate after 8 weeks in capsules coats and that was associated with the decreasing in mortality percentage (3.33 %) and sprouting percent (15.00 %) for the same treatment.

Sodium alginate at 3 % and calcium chloride at 100 mM had found to be optimum concentration for the production of uniform synthetic seed of *Vitex negundo* L. (Ahmad and Anis, 2010).

Table 6: Effect of different concentrations of sodium alginate and calcium chloride on conservation of *Odontonema cuspidatum*

Calcium chloride (g/100ml) \ Sodium alginate (%)	Mortality (%)				Inhibition (%)				Sprouting (%)			
	3	4	5	Mean (A)	3	4	5	Mean (A)	3	4	5	Mean (A)
1.1	0.00	0.00	3.33	**1.11**	0.00	0.00	81.67	**27.22**	100.0	100.0	15.00	**71.67**
2.2	0.00	0.00	18.33	**6.11**	0.00	0.00	70.00	**23.33**	100.0	100.0	11.67	**70.56**
3.3	0.00	5.00	38.33	**14.44**	0.00	8.33	55.00	**21.11**	100.0	86.67	6.67	**64.44**
Mean (B)	**0.00**	**1.67**	**20.00**		**0.00**	**2.78**	**68. 89**		**100.0**	**95.56**	**11.11**	
$LSD_{0.05}$ for												
Sodium lginate(A)			**1.67**				**2.20**				**1.44**	
Calcium chloride(B)			**1.67**				**2.20**				**1.44**	
(AxB)			**2.89**				**3.82**				**2.50**	

Effect of MS medium strength in capsules coat and culture medium on conservation:

Data presented in Table (7) showed that the shoot tips which were coated with different media and cultured on half-strength MS medium with sugar gave zero level of mortality and inhibition percentage for the most capsules coat, giving no significant differences between them, but the sprouting percentage was 100 % for the most capsules coat.

However, mortality percentage recorded low values when the encapsulated shoot tips were cultured on half-strength MS medium without sugar. The shoot tips, which were coated with half-strength MS medium with sucrose resulted in inhibition percentage of 96.33 %, with zero sprouting. Addition of MS nutrients in capsules coat improved encapsulated shoot tips regrowth after the storage periods. These results are in line with that obtained on *Saintpaulia ionantha* (Daud *et al*, 2008). Moreover, $CaCl_2.2H_2O$ solution gave the optimal time of hardening process.

Table 7: Effect of medium in capsules coat and culture medium at light and 28℃ on conservation of *Odontonema cuspidatum.*

Capsules Coat \ Culture media	½ Ms with sucrose			½ MS without sucrose		
	Mortality (%)	**Inhibition (%)**	**Sprouting (%)**	**Mortality (%)**	**Inhibition (%)**	**Sprouting (%)**
Full Ms + sucrose	0.00	0.00	100.0	0.00	11.11	88.89
Full MS without sucrose	0.00	0.00	100.0	0.00	11.11	88.89
½ Ms + sucrose	0.00	0.00	100.0	3.67	96.33	0.00
½ MS without sucrose	0.41	0.41	99.18	0.00	3.67	96.33
$LSD_{0.05}$	**0.60**	**0.60**	**1.20**	**5.35**	**20.66**	**19.84**

Effect of calcium chloride at different exposure periods (at two layers) in capsules coat and storage periods on conservation:

Addition of calcium alginate at two layers improved the encapsulated shoot tips inhibition during storage for long periods. The conservation of synthetic seeds decreased gradually with increasing storage period.

Regardless data in Table (8), encapsulated shoot tips were coated with two layers and cultured on half-strength MS medium without sucrose as showed good results on conservation. It can be noticed that increasing the exposure periods to calcium chloride induced the percentage of inhibited shoot tips and reduced the sprouting percentage. Concerning the different periods of storage at the same incubation condition of temperature and light, there were significant differences between these treatments during the storage periods for 2, 4 and 8 weeks on the percentage of inhibition and

sprouting. Concerning the interaction between the exposure periods to calcium chloride and the storage periods, it was found that the highest percentage of inhibited shoot tip was 91.67% and gave the lowest sprouting percentage (8.33 %) at 15 min of exposure to calcium chloride after the longest period of conservation (eight weeks).

Pinker and Abdel-Rahman, (2005) reported that second layer of Ca-alginate with water reduced the shoot formation recording as 73% of the encapsulated nodal segments in *Dendranthema x grandiflora*.

Table 8: Effect of calcium chloride with different exposure times (at two layers) in capsules coat and storage periods (weeks) on conservation of *Odontonema cuspidatum*.

Exposure times (min) \ Storage periods (weeks)	Inhibition (%)				Sprouting (%)			
	2	4	8	Mean(A)	2	4	8	Mean (A)
5	81.00	71.67	48.33	**67.00**	19.00	28.33	51.67	**33.00**
10	91.67	84.00	66.67	**80.78**	8.33	16.00	33.33	**19.22**
15	97.00	92.00	91.67	**93.56**	3.00	8.00	8.33	**6.44**
Mean (B)	89.89	82.56	68.89		10.11	17.44	31.11	
LSD 0.05 for								
Exposure times (min) (A)				**1.20**				**1.08**
Storage periods(weeks) (B)				**1.20**				**1.08**
(AxB)				**2.08**				**1.87**

References

Ahmad, N. and M. Anis, 2010. Direct plant regeneration from encapsulated nodal segments of *Vitex negundo*. Biologia Plantarum, 54 (4): 748-752.

Cheruvathur, M. K. and T. D. Thomas, 2014. High frequency multiple shoot induction from nodal segments and rhinacanthin production in the medicinal shrub *Rhinacanthus nasutus* (L.) Kurz. Plant Growth Regul, (74):47–54.

Daud, N., R.M. Taha and N.A. Hasbullah, 2008. Artificial seed production from encapsulated micro shoots of *Saintpaulia ionantha* Wendl. (African violet). J. Appl.Sci., 8(24): 4662-4667.

Elangomathavan, R., S. Hariharan, P. Kalaivanan, and B.S. Nancy, 2017a. Propagation of *Rhinacanthus nasutus* (L.) Kurz., through encapsulated shoot tips and nodal segments for germplasm exchange and distribution. Int J Pharm Bio Sci., 8(1): 98 – 105.

Elangomathavan, R., B.S. Nancy, S. Hariharan, and P. Kalaivanan, 2017b. Encapsulation of shoot tips and nodal segments of *Cleistanthus collinus* for short term storage and germplasm exchange. Int. J. Current Res. Bio. Med., 2(1): 21-32.

Girija, S., A. Ganapathi, and G. Vengadesan, 1999. Micropropagation of *Crossandra infundibuliformis* (L.) Nees. Sci. Hort., (82): 331-337.

Gunasekaran, U., 2014. Callus induction and plant regeneration studies of *Clinacanthus nutans* (sabah snake grass). Bachelor of Science (Hons) Biotechnology, the Department of Biological Science, Faculty of Science ,Universiti Tunku Abdul Rahman, 87 pp.

Hassan, S., N. Begum, R. Sultana and R. Khatun, 2011. *In vitro* shoot proliferation and plant regeneration of *Phlogacanthus thyrsiflorus* Nees. a rare medicinal shrub of Bangladesh. Plant Tissue Cult. & Biotech, 21(2): 135-141.

Karuppusamy, S. and K. Kalimuthu, 2010. Rapid *in vitro* multiplication and plant regeneration from nodal explants of *Andrographis neesiana*: A valuable endemic medicinal plant. Advances in Biological Research, 4 (4): 211-216.

Kumar, M.S. and S.C. Nandi, 2015. High frequency plant regeneration with histological analysis of organogenic callus from internode explants of *Asteracantha longifolia* Nees. J. Gen. Eng. Bio.,12.

Lakshmanan, P. and J. J. Gabriel, 2015. *In vitro* regeneration of *Ruellia tuberosa* L. (Acanthaceae) through direct organogenesis. Int. J. Recent Sci. Res., 6 (5): 3988-3990.

Mohammed, A., K.K. Chiruvella, and R.G. Ghanta, 2016. *In vitro* plant regeneration, flowering and fruiting from nodal explants of *Andrographis lineata* Nees (Acanthaceae). J. Crop. Sci. Bio., 19(3): 195 – 202.

Pinker, I. and S.S.A. Abdel-Rahman, 2005. Artificial seeds for propagation of *Dendranthema x grandiflora* (RAMAT.). Propag. Ornament. Plants, 5(4): 186-191.

Sharma, S. and A. Shahzad, 2012. Encapsulation technology for short-term storage and conservation of a woody climber, *Decalepis hamiltonii* Wight and Arn. Plant Cell Tiss Organ Cult, (111):191–198.

Sharma, V., 2014. *In vitro* propagation of endangered orchid taxa using alginate encapsulated protocorm like bodies (PLBs). Nature Envir. Poll. Tech., 13(2).

Snedecor, G.W. and W.G. Cochuran, 1989. Statistical Methods low a State Aniv. Press, Ames, Iows, U.S.A., 201-236.

Taha, R.M., N.A. Hasbullah and A. Awal, 2009. Production of synthetic seeds from micro shoots and somatic embryos of *Gerbera jamesonii* Bolus EX. HOOK F. Acta Hort.,829(12):91-98.

Wang, W., S. Wang, X. Wu and F. Chen, 2007. High frequency plantlet regeneration from callus and artificial seed production of rock plant *Pogonatherum paniceum* (Lam.) Hack. (Poaceae). Sci. Hort., 113(2):196-201.