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IN VITRO PROPAGATION OF CHUNGAH (*CARALLUMA TUBERCULATA* N.E. BROWN)

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Abstract

In vitro propagation of *Caralluma tuberculata* (Chungah) was developed from shoot tip and meristem explants. *C. tuberculata* is an imperative medicinal plant comprising antidiabetic and anticancer properties. The explants were inoculated on Murashige and Skoog (MS) medium containing different plant growth regulators. Presence of BA or Kin alone in the MS medium did not favor regeneration of shoot from both explants. However, addition of 2,4-dichlorophenoxy acetic acid (2,4-D), gibberellic acid (GA_3) and thidiazuron (TDZ) along with 6-benzyl amino purine (BA) or kinetin (Kin) in the medium exhibited significant percentage response, number of shoots per explant and shoot length. Maximum shooting response (53.3±5.77% from meristem and shoot tip explants each) with highest number of shoots per explant (5.33±2.08 and 5.6±2.52 from meristem and shoot tip explants, respectively) were observed at 13.32 µmol BA along with 2.26 µmol 2,4-D, 2.89 µmol GA_3 and $9.08x10^{-3}$ µmol TDZ. Replacing BA with kin showed less shoot regeneration response and number of shoots per explant, however, shoots length markedly increased in the presence of Kin. The regenerated plants were successfully rooted and acclimatized in ex vitro condition. The protocol described here can be used for fast multiplication of this endangered herb and genetic transformation.

Key words: *In vitro*, *Caralluma tuberculata,* Antidiabetic, Anticancer.

Introduction

The genus *Caralluma* (Asclepiadaceae) comprises about 260 species that are succulent, perennial and branched herbs. The species are distributed in Middle East, Africa, Spain and some other countries. In Pakistan, two species *C. tuberculata* (N.E. Brown) and *C. edulis* (Edgew) grow wild in the scattered foot-hill areas of Suleiman and Hindukush ranges in Baluchistan, Punjab and Khyber Paktunkhwa. The genus *Caralluma* has great industrial and pharmaceutical interest because it contains variety of pregnane glycosides e.g. russelioside A-D (Al-Yahya *et al*., 2000); russeliosides E-G (Abdel-Sattar *et al*., 2007); carumbellosides I and II (Lin *et al*., 1994); carumbellosides III, IV and V (Qiu *et al*., 1997); lasianthoside-A and B (Qiu *et al*., 1999); megastigmane glycosides and flavone glycosides 3 and 4 (Bader *et al*., 2003). These phytochemical compounds possess antitumor (Qiu *et al*., 1999); platelet pro- aggregating (Piacente *et al*., 1998); anti-fungal (Hu *et al*., 1999); digitalis receptor binding (Templeton *et al*., 1993); and antiulcer properties (Zakaria *et al*., 2002). The plants also have significant anti-inflammatory, antioxidant and antitumor activities (Zakaria *et al*., 2001). Despite its importance in modern era, *Caralluma* species are also prescribed as herbal drug from long to control diabetic ailments in Ayurvedic system of medicine. Furthermore, *Caralluma* species are used to treat pain, fever, appetite suppressant, and also stimulate central nervous system. Native populaces collect *Caralluma* (Chungah) plant material from the wild to use it as food, medicine and trade. This over collection has resulted in gradually vanishing of this specie from natural biome. There is urgent need to conserve the plant by developing protocols for its multiplication both through conventional and modern plant tissue culture techniques.

Caralluma species could not attain much attention because difficulties in culturing process, release of phenolics and latex that hinder callus induction and shoot regeneration. Recently a protocol for shoot organogenesis of *C. sarkariae* (Sreelatha *et al*., 2009; Ugraiah *et al*., 2011) and *C. adscendens* (Aruna *et al*., 2009) was developed using MS medium enriched with plant growth regulators. There is no report of regeneration of *C. tuberculata* through direct organogenesis. The present study was undertaken to develop an efficient procedure for *In vitro* regeneration of *C. tuberculata* from shoot tip and meristem explants through organogenesis on different combinations and concentrations of plant growth regulators.

Materials and Methods

Plant material used for this study was obtained from Quetta (Balochistan, Pakistan) and was identified by Prof. Dr. Mir Ajab Khan Department of Plant Sciences, Quaid-i-Azam University Islamabad, Pakistan. The plant material was multiplied in earthen pots in green house for continuous supply of explants.

Surface sterilization: Before the experiment, the plants were initially washed under running tap water for 30 min to remove all the adhering dust particles following washing with liquid detergent (Titron X 100) for 15 min. The plant material was rinsed with distilled water succeeding treatment with bevistin (a fungicide) for 20-30 min to remove fungus and again rinsed with distilled water. Under aseptic conditions, the explants were treated with 0.1% $HgCl_2$ (w/v) solution for 5-10 min. The explants were thoroughly rinsed (5x5 min) with sterilized distilled water to remove traces of $HgCl_2$. Thereafter,

meristem and shoot tip portions (5-10 mm^2) were isolated aseptically and used as explants.

Culture media and culture conditions: The MS medium (Murashige & Skoog, 1962) was used as basal medium in all the experiments. Basal medium was supplemented with BA (6-benzyl amino purine) or Kin (kinetin), alone or in combination with 2,4-D (2,4-dichlorophenoxy acetic acid), GA_3 (gibberellic acid) and TDZ (thidiazuron), at different concentrations. In all the culture media, sucrose (3%) was added as carbon source. The pH of media was adjusted at 5.7±0.2 using 0.1N KOH or 1N HCl before autoclaving at 121°C under pressure of 103.42 KPa for 20 min. The media was solidified with 0.7% noble agar (Merck). All the cultures were maintained in growth room at a temperature of 25±2°Ċ. The source of illumination consisted of 4 feet long 40 W Philips tubes, day light fluorescent lamps and incandescent bulb (25 W). The intensity of illumination was 3500 lux at the level of cultures, and all cultures were provided with 16 h light photoperiod.

Under aseptic condition, meristem and shoot tip explants were inoculated on the MS medium. The inoculation medium without growth regulator was used as control. A total of 15 explants (meristem and shoot tip each) were inoculated independently. Percentage response, shoot length and number of shoots emerged per explant were recorded after 40 days of culture. Afterward, the shoots were separated from the base and cultured on rooting medium comprised of half strength MS medium supplemented with indol butyric acid (IBA) or indol 3-acetic acid (IAA). Once a significant rooting system was developed, the plants were removed from the flasks, rinsed under running tap water to remove media and planted in plastic pots containing peat moss and sand (1:1). The plastic pots were also covered with transparent polythene bags to retain humidity that was gradually removed after 10 days. The survived plants were shifted to earthen pots and shifted to natural conditions.

Statistical analysis: Each concentration and combination was considered as an independent experiments. A total of 15 explants (shoot tip and meristem independently) were cultured in each experiment. Shooting response, number of shoots per explant and shoot length was recorded after 40 days of culture. To study rooting response, 10 plants were cultured at different concentrations of IBA and IAA. Percent rooting response and number of roots per plant were calculated after 30 days of culture. One-way analysis of variance (ANOVA) was performed for shoots and root regeneration data analysis. Data were processed statistically with SAS v.9 software. Least significant difference (*lsd*) test was applied at 5% probability level for the comparison of treatment means. The results are expressed as mean ± standard error for these experiments.

Results and Discussion

Organogenesis requires the re-initiation of cell division by the application of appropriate plant growth regulators (Benson, 2000). Only a few reports of latex-producing plants have been documented, including *Hevea* (Wilson *et al*., 1976), *Papaver* (Nessler, 1982) and *Asclepias* (Dunbar *et al*., 1986). For organogenesis of *C. tuberculata* from meristem and shoot tip explants, BA (6-benzyl amino purine) was found better as compared with Kin (kinetin). However, addition of TDZ (thidiazuron), 2,4-D (2,4 dichloro phenoxy acetic acid) and GA_3 (gibberellic acid) were obligatory for optimum results. Absence of TDZ from culturing media drastically affected percent shooting response and number of shoots per explant (unpublished data). Therefore, we incorporated TDZ quite at low concentration ($9.08x10^{-3}$ µmol) in the medium with other growth regulators. The treatment of TDZ normally involves in morphogenesis of the competent cells into shoots (Murthy & Saxena, 1998). The transition effect of TDZ for embryogenesis and organogenesis has been reported in many plant species i.e., *Arachis hypogaea* (Chengalaryan *et al*., 1997), *Paspalum scrobiculatum* (Vikrant & Rashid, 2002). However, TDZ in combination with auxin (2,4-D), effectiveness to produce shoots increases many times (Chandrasekhar *et al*., 2006). While, the stimulatory effect of GA_3 in organogenesis have been reported by Hita *et al*., (1997) and Rudus *et al*., (2002). In all the cases, formation of callus, at the base of shoot tip explants or conversion of meristem explant into callus, slackened the shooting response, thereof, number of shoots per explant and shoot length. It was also observed that shoot tip explants responded better in terms of percentage response and number of shoots per explant as compared with meristem explants. These results are consistent with those of many reports, that the source of explants, explants type and age are important factors in determining *In vitro* responses. This may suggest that levels of endogenous hormones or their hormone responsivity might vary between organs (Sharma & Rajam, 1995; Zia *et al*., 2010).

The percentages of regeneration 43.3 and 46.6 were recorded when meristem and shoot tip explants, respectively, were cultured on MS medium containing 8.88 µmol BAP singly in the culturing medium (Table 1). At this concentration, 2.3 shoots per shoot tip explant and 1.3 shoots per meristem explant were observed with average shoot length 3.6 and 4.6 cm, respectively. The same concentration was also found effective for induction of multiple shoots from nodal segments of *C. adscendens* (Aruna *et al*., 2009) and *C. bhupenderiana sarkaria* (Ugraiah *et al*., 2011). Wang & Bao (2007) also observed that combinations of 2,4-D or NAA with BAP were essential for maximum production of calli in *Viola wittrockiana*. Combination of BAP with GA_3 and TDZ was not much persuading, however, addition of 2,4-D along with these regulators accelerated the outcomes in

different fashions. Application of 8.88 µmol BA with 2,4-D (2.26 µmol and 4.52 µmol), GA_3 (2.89 µmol and 5.78 µmol) and TDZ ($9.08x10^{-3}$ µmol) gave 33% initiation response from meristem explant, while shoot tip explant responded 30 to 46% (Fig. 1a & b). The highest average number of shoots viz. 2.66±1.15 and 3.3±0.58 were recorded from meristems and shoot tip explants, respectively (Table 1). Maximum mean shoot length regenerated from meristem explants was 5.3±0.58 cm while 4.3±0.58 cm from shoot tip explants. These results are consistent with previous reports that explants from different organs resulted in different organogenic responses (Petersen *et al*., 1999; de Paiva-Neto *et al*., 2003; Osuna *et al*., 2006). The shoot length response from meristem derived shoots was comparatively better than that of shoot tip explants at all the combinations. The use of plant growth regulators in combination has been extensively reported. Application of many growth regulators in the medium cumulatively affect in number of fashions; presence of cytokinin (BA) induces the shoots, GA_3 favor elongation through increased cell division, and auxin (2,4-D) for cell division while inclusion of TDZ also modulates endogenous growth hormones (Thomas & Shankar, 2009). Combination of BA, Kin and NAA has been found effective for shoot induction from *Caralluma sarkariae* explants (Aruna *et al*., 2009).

Increase in the concentration of BA (13.32 µmol) along with 2,4-D, GA_3 and TDZ enhanced shooting response (%) as well as number of shoots per explant. The response from meristem explants varied from 36% to 53%, and that of shoot tip explant 46% to 56%. The maximum shooting response 53% was recorded on 13.32 µmol BA + 2.26 µmol 2,4-D + 2.89 µmol GA_3 + $9.08x10^{-3}$ µmol TDZ in MS medium. This treatment also produced the highest average number of shoots from meristem (5.33) and shoot tip (5.60) explants. However, average length of shoots from meristem and shoot tip explants was 3.0 and 3.3 cm, respectively. Further increase in BA concentration decreased the rate of shooting response as well as the number of shoots produced per explant. Though, the length of shoots increased considerably by increasing the BA concentration. On MS medium supplemented with 17.76 µmol BA, 2.26 µmol 2,4-D, 5.78 µmol GA_3 and $9.08x10^{-3}$ µmol TDZ, the shooting response decreased up to 26% from meristem explant and 36% from shoot tip explant. The meristem explants produced 3.0 shoots as compared to 2.3 shoots produced from shoot explants. Combination of cytokinin (BA, Kin or TDZ) with auxin has been reported for shoot induction for many species of *Asclepiadaceae* (Thomas & Shankar, 2009; Thomas & Philip, 2005), nevertheless, percent response and induction of multiple shoots depends upon plant species, explant used, and concentration and combination of plant growth regulators. The cells can produce shoots under stresses including auxins alone or in combination with cytokinins (Gaj, 2004). Although, induction of shoots from cells is sequential process but still exogenous supply of plant growth regulators especially auxin derive the cell to produce shoots healthier way (Jimenez, 2001). The exogenous supply also modulates internal concentration of PGRs or PGR synthesizing enzymes (Thorpe, 2000; Gaspar *et al*., 2003; Gazzarrini & McCourt, 2003) involving modulation of endogenous plant hormone concentrations resulting in shoots development.

The response of Kin was not as profound as that of BA in relation to percentage response, average number of shoots per explant and average length of shoot (Table 2). No shoot formation was observed from both explants when explants were cultured on MS medium containing 9.3 µmol Kin. Among all the Kin combinations, shoot induction varied from 23% to 53%. With the treatment of 9.3 µmol Kin, 2.26 µmol 2,4-D along with 5.78 µmol GA_3 and $9.08x10^{-3}$ µmol TDZ, the average number of shoots from meristem explants was 3.3, and 3.0 from shoot tip explant (Fig. 1c & d). At all the combinations of Kin with other growth regulators, percentage shooting response and mean number of shoots were less while mean shoot length was higher irrespective to explant type. Inclusion of kin in MS medium for organogenesis of *C. bhupenderiana sarkaria* did not produce better percent response and number of shoots per explant (Ugraiah *et al*., 2009).

The regenerated shoots cultured on half strength MS medium (control) failed to root. Addition of 5.07 µmol IAA (indol 3-acetic acid) in half strength MS medium resulted in 3.0±0.15 mean number of roots per plantlet (Table 3) after 30 days of culture (Fig. 1e). The same findings are also reported by Ugraiah *et al*., (2009). Environmental conditions for ex vitro growth are quite different from those used for *In vitro* cultivation. During acclimatization process, water loss from plantlets in ex vitro conditions is a major problem (Hazarika, 2003, 2006). The *In vitro* regenerated plantlets were acclimatized ex vitro in pots covered with translucent plastic bags to ensure high humidity (Bibi *et al*., 2011). The use of this procedure during the acclimatization phase ensured that most plantlets transplanted to ex vitro conditions continued to grow vigorously (Fig. 1f). When the plastic bags were removed after two weeks, a mean of 80% of the plantlets survived. The acclimatized plants propagated and produced more number of shoots. It is important to note that the morphology of the *In vitro* regenerated plantlets showed a true to type rosette growth habit, both *In vitro* and when transferred to ex vitro growth conditions.

The present study describes for the first time protocol for the production through tissue culture of plantlets of *C. tubercaulata*, an endangered species with potential industrial application. This protocol could be used to clone plantlets growing from native plant seeds and therefore may represent an important tool for the preservation of biodiversity and provide valuable material for the long-term storage of plant genetic resources. Moreover, this protocol can also be used for genetic transformation.

Table 1. The effect of BA, 2, 4-D, GA_3 and TDZ on shoot organogenesis from meristem and shoot tip explants of *Caralluma tubercaulota*.

Plant growth regulator (µmol)				Shooting response (%)		No. of shoots per explant		Mean shoot length (cm)	
BA	2,4-D	GA3	TDZ	Meristem	Shoot tip	Meristem	Shoot tip	Meristem	Shoot tip
8.88	-	-	-	43.3 ± 5.77b	46.6 ± 11.55b	1.33 ± 0.58e	2.3 ± 0.58cd	4.6 ± 0.58b	3.6 ± 0.58cd
8.88	-	2.89	9.08×10^{-3}	36.6 ± 5.77c	46.6 ± 15.28ab	1.33 ± 0.58e	2.3 ± 0.58cd	4.6 ± 0.58b	3.6 ± 0.58cd
8.88	-	5.78	9.08×10^{-3}	36.6 ± 5.77c	43.3 ±5.77bc	2 ± 1c	2 ± 1	4 ± 1bcd	3.6 ± 0.58cd
8.88	2.26	2.89	9.08×10^{-3}	33.3 ± 5.77cd	30 ± 10d	2 ± 1c	2.3 ± 0.58cd	4.3 ± 0.58bc	4 ± 1.73abc
8.88	2.26	5.78	9.8×10^{-3}	33.3 ± 5.77cd	40 ± 0cd	2.66 ± 1.15cd	3.3 ± 0.58bcd	3.3 ± 0.58cde	4 ± 0bc
8.88	4.52	2.89	9.08×10^{-3}	33.3 ± 5.77cd	46.6 ± 5.77bc	1.3 ± 0.58e	1.3 ± 0.58d	5.3 ± 0.58a	4.3 ± 0.58b
8.88	4.52	5.78	9.08×10^{-3}	33.3 ± 5.77cd	36.63 ± 5.77c	1.66 ± 1.15de	1.6 ± 1.15d	4 ± 1bc	4 ± 1bc
13.32	2.26	2.89	9.08×10^{-3}	53.3 ± 5.77a	53.3 ± 5.77ab	5.33 ± 2.08a	5.6 ± 2.52a	3 ± 0de	3.3 ± 0.58d
13.32	2.26	5.78	9.08×10^{-3}	36.6 ± 5.77c	46.6 ± 5.77bc	4.66 ± 1.53ab	5.3 ± 2.08ab	3.3 ± 0.58cde	3.3 ± 0.58d
13.32	4.52	2.89	9.08×10^{-3}	40 ± 10ab	53.3 ± 5.77ab	4.66 ± 1.53ab	4.3 ± 1.53bc	3.6 ± 0.58cd	3.3 ± 0.58d
13.32	4.52	5.78	9.08×10^{-3}	43.3 ± 11.55b	56.6 ± 15.28a	4.66 ± 1.53ab	5.3 ± 2.08ab	3.3 ± 0.58cde	3.3 ± 0.58d
17.76	2.26	2.89	9.08×10^{-3}	26.6 ± 5.77d	36.6 ± 5.77c	3 ± 1b	2.3 ± 0.58cd	3.6 ± 0.58cd	4 ± 0bc
17.76	2.26	5.78	9.08×10^{-3}	26.6 ± 5.77d	36.6 ± 5.77c	2.66 ± 1.15cd	3 ± 1bcd	4 ± 1bc	4.3 ± 0.58b
17.76	4.52	2.89	9.08×10^{-3}	33.3 ± 5.77cd	53.3 ± 5.77ab	1.66 ± 1.15de	1.6 ± 1.15d	4.3 ± 0.58bc	4.6 ± 0.58a
17.76	4.52	5.78	9.08×10^{-3}	30 ± 10cd	40 ± 10bcd	2.66 ± 1.53cd	3 ± 1bcd	4.3 ± 1.53abc	4.3 ± 0.58b

± Shows value of standard deviation from treatment Different letter(s) after data within column represent statistically difference among treatment means at $p<0.05$ using LSD test

Table 2. The effect of Kinetin, 2, 4-D, GA_3 and TDZ on shoot organogenesis from meristem and shoot tip explants of *Caralluma tubercaulota*.

Plant growth regulator (µmol)				Shooting response (%)		No. of shoots per explant		Mean shoot length (cm)	
Kin	2,4-D	GA3	TDZ	Meristem	Shoot tip	Meristem	Shoot tip	Meristem	Shoot tip
9.3	-	-	-	0	0	0	0	0	0
9.3	-	2.89	9.08×10^{-3}	30 ± 0c	36.6 ± 5.77cd	1.33 ± 0.58d	2 ± 1cd	5.3 ± 0.58a	4.6 ±0.58a
9.3	-	5.78	9.08×10^{-3}	23.3 ± 5.77d	36.6 ± 5.77cd	2 ± 1c	2.6 ± 1.15bc	5 ± 1ab	3.6 ± 0.58c
9.3	2.26	2.89	9.08×10^{-3}	16.6 ± 5.77e	26.6 ± 1.15d	2.6 ± 1.15b	2.3 ± 1.53bcd	3.3 ± 0.58e	3.6 ± 1.15bcd
9.3	2.26	5.78	9.8×10^{-3}	26.6 ± 5.77cd	43.3 ± 5.77bc	3.33 ± 0.58a	3 ± 1b	4 ± 1cde	4.3 ± 0.58bc
9.3	4.52	2.89	9.08×10^{-3}	16.6 ± 5.77e	36.6 ± 5.77cd	1.33 ± 0.58	1.3 ± 0.58d	4.6 ± 1.15abc	4.4 ± 0.58b
9.3	4.52	5.78	9.08×10^{-3}	16.6 ± 5.77e	53.3 ± 5.77a	1.66 ± 1.15cd	2 ± 1cd	4.6 ± 0.58bc	4.6 ± 1.15ab
13.94	2.26	2.89	9.08×10^{-3}	36.6 ± 5.77b	46.6 ± 5.77abc	2.33 ± 0.58bc	2.6 ± 1.15bc	3.6 ± 0.58de	3.6 ± 0.58c
13.94	2.26	5.78	9.08×10^{-3}	46.6 ± 5.77a	53.3 ± 5.77a	3 ± 1ab	3.6 ± 1.53a	3.3 ± 0.58e	3.3 ± 0.58d
13.94	4.52	2.89	9.08×10^{-3}	16.6 ± 5.77e	26.6 ± 5.77d	1.33 ± 0.58d	2.3 ± 0.58bcd	4.6 ± 0.58bc	4.3 ± 0.58bc
13.94	4.852	5.78	9.08×10^{-3}	30 ± 10bc	53.3 ± 5.77a	1.66 ± 0.58cd	2 ± 1cd	4.3 ± 0.58cd	4.3 ± 0.58bc
18.6	2.26	2.89	9.08×10^{-3}	46.6 ± 5.77a	50 ± 10ab	2.66 ± 1.15b	3.6 ± 1.53a	4.6 ± 1.53abc	4.3 ± 0.58bc
18.6	2.26	5.78	9.08×10^{-3}	36.6 ± 5.77b	43.3 ± 5.77bc	3 ± 1ab	3 ± 1b	5 ± 1.73ab	4.3 ± 0.58bc
18.6	4.52	2.89	9.08×10^{-3}	36.6 ± 5.77b	33.3 ± 5.77bc	1.66 ± 1.15cd	2.6 ± 1.15bc	4.3 ± 0.58cd	4.6 ± 0.58a
18.6	4.52	5.78	9.08×10^{-3}	16.6 ± 5.77e	46.6 ± 5.77abc	1.33 ± 0.58d	2 ± 1cd	4.6 ± 0.58bc	4.3 ± 0.58bc

± Shows value of standard deviation from treatment Different letter(s) after data within column represent statistically difference among treatment means at $p<0.05$ using LSD test

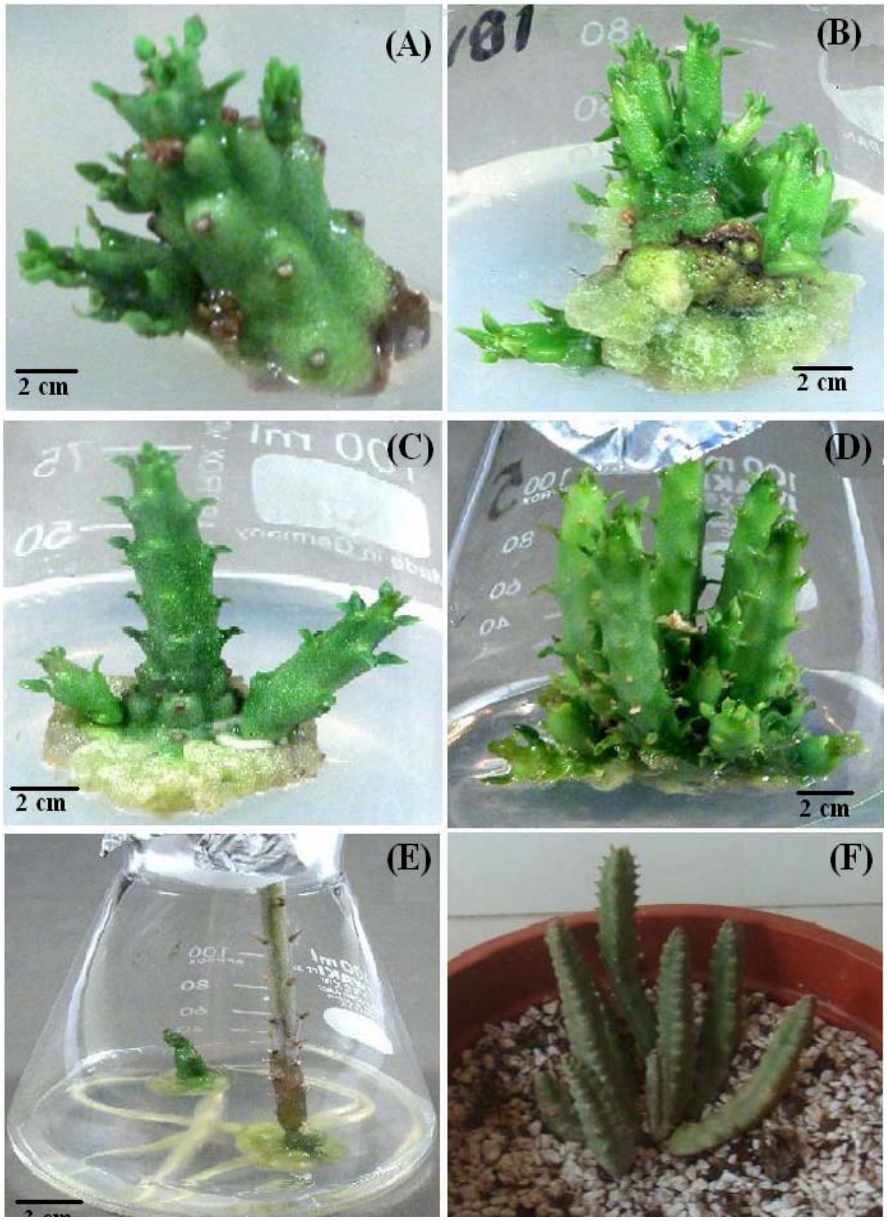

Fig. 1. Shoot Organogenesis from shoot tip and meristem explants of *Caralluma tuberculata*; (A & B) Shooting response on 13.32 µmol BA, 2.26 µmol 2,4-D, 2.89 µmol GA_3 and $9.08x10^{-3}$ TDZ from shoot tip and meristem explant, respectively; (C & D) Shooting response on 13.94 µmol Kin, 2.26 µmol 2,4-D, 5.78 µmol GA_3 and $9.08x10^{-3}$ TDZ from meristem and shoot tip explant, respectively; (E) Rooting response on 5.07 µmol IAA; (F) Acclimatization of *In vitro* grown *Caralluma tuberculata*.

Table 3. Effect of IAA and IBA on root induction of *In vitro* grown plantlets of *C. tuberculata*

IAA (µmol)	IBA (µmol)	Response (%)	No. of Roots/plant
2.54	0.0	22.5 ± 0.5c	0.63 ± 0.15c
5.07	0.0	50.16 ± 1.6a	3.06 ± 0.15a
7.61	0.0	35.16 ± 1.0b	2.0 ± 0.1b
0.0	2.46	19.2 ± 0.3d	0.5 ± 0.1c
0.0	4.92	24.03 ± 0.9c	0.6 ± 0.1c
0.0	7.38	22.33 ± 1.5c	0.7 ± 0.2c

± Shows value of standard deviation from treatment mean.

Different letter(s) after data within a column represent statistically significant difference among treatment means at $p \leq 0.05$ using *lsd* test

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