British Biotechnology Journal
4(2): 164-172, 2014

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In vitro Propagation of *Caralluma diffusa* (Wight) N.E.Br

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Authors' contributions

All the authors have cordially supported to the work and preparation of manuscript. Authors TS and RP have designed the entire study and protocols with interpretations of the results and prepared the first draft of the manuscript. Author KK managed the analyses of the study and computational work respectively. Author KK guided in the entire research and documented the final draft of the manuscript. All the authors have read and approved the final manuscript.

Original Research Article

Received 19th October 2013
Accepted 25th November 2013
Published 27th December 2013

ABSTRACT

An efficient protocol is described for the rapid *in vitro* multiplication of an endangered medicinal plant, *Caralluma diffusa*, via enhanced axillary bud proliferation from nodal explants collected from young shoots of six-months-old plant. The physiological effects of growth regulators Benzylamino purine (BAP), Thidiazuron (TDZ), Kinetin (KIN), α-Naphthalene acetic acid (NAA) full strength of Murashige and Skoog's (MS) medium on *in vitro* propagation were investigated. The highest number of shoots in initiation (4.16±0.30) and in subculture (17.33±0.33) and the maximum average shoot length (4.17±0.03cm) were recorded on MS medium supplemented with BAP (4.44 µM) and TDZ (0.90 µM) at pH 5.8. Rooting was best achieved on MS medium augmented with NAA (5.37 µM). The plantlets regenerated *in vitro* with well-developed shoot and roots were successfully established in pots containing decomposed coir waste, vermiculite and garden soil (1:1:1ratio) and grown in a shade house with 93.83% survival rate.

Keywords: In vitro; micropropagation; Caralluma diffusa; medicinal plant; endangered.

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1. INTRODUCTION

The genus *Caralluma* comprises about 200 genera and 2500 species. It belongs to sub tribe Stapeliinae (tribe Ceropegiae, sub family Asclepiadoideae and family Apocynaceae), which has its Centre of origin in East Africa [1]. The member of this genus is a small erect fleshy plant which has four grooved round shaped stems devoid of leaves with small flowers of several dark colours. The species of *Caralluma* found in India are edible and forms a part of the traditional medicine system of the country [2]. In India, the genus is represented by 13 species and 7 varieties, out of which 11 species are solely endemic to South India [3]. *Caralluma,* found in dry regions of the world, has paramount medicinal importance and has significant anti-inflammatory and antitumor activity [4,5]. The pregnane glycosides isolated from *C. umbellata* have shown significant anti-inflammatory property [6]. *Caralluma* species are commonly used in the treatment of rheumatism, diabetes, leprosy, antipyretic and anthelmintic, for tumor, fungal diseases, snake, scorpion bite and analgesic activity [7,8]. *C. adscendens* have significant analgesic and antimutagenic properties which promote immune system [9,10]. It was observed that the administration of *C. sinaica*in different doses to healthy animals can cause significant decrease in glucose level [11]. In another report, it was observed that *C. fimbriata*, can be used in weight reduction [12]. *C. edulis*is known for its antidiabetic properties [13] and other *Caralluma* species for their anti hyperglycemic activity [14]. The extracts of *C. attenuate and C. edulis*had hypoglycemic properties and provide synergistic effect in combination with the phlorizin extract which beneficially modify glucose transport, blood and urine glucose levels, blood insulin levels and helps in weight loss. Plants of *C. tuberculata* have been extensively used for the paralysis and joints pain and fever [15]. *C. tuberculata* stem juice is used as bitter tonic, febrifuge, carminative, in rheumatism, is consumed as vegetable [16]and as anti parasitic medicine [17]. *C. umbellata*is used in Indian traditional medicine system for stomach disorder and abdominal pains [18]. Pharmacological Studies revealed that *C. arabica* extract has anti-nociceptive, anti-gastric ulcer, cytoprotective, and anti-inflammatory properties [19].

Caralluma diffusa Wight (syn. *Boucerosia diffusa* (Wight) N.E.Br)is a rare and an endemic medicinally important plant belonging to the family Asclepiadaceae. Distribution of this species as Deccan, arid rocky hills near Coimbatore at an elevation of about 600m [20]. Asclepiadaceae plant species have poor seed germination and viability [21]. The Flora of Tamil Nadu states that *C. diffusa* distribution is only from Coimbatore district in Tamil Nadu and ecological status is mentioned as rare and threatened species [22]. This species is rediscovered after 160 years from Coimbatore district [23]. There has been over exploitation for commercial purpose and indiscriminate collection of *C. diffusa* by the local peoples because of its therapeutic uses and this has lead to severe biotic pressure. This species is naturally reproduced by clonal propagation, so minimum number of plants survived in the ecosystem. Propagation through seed is hampered by non viability. In order to meet the increasing demand for *C. diffusa*, present study was aimed to develop a reproduceable protocol for mass propagation of the plant in order to conserve it in the wild.

2. MATERIALS AND METHODS

2.1 Plant Material and Surface Sterilization

Plants of *C. diffusa* were collected from Madukarai hills, Coimbatore District, Tamil Nadu, India and maintained in earthen pots in shade house at Government Arts College, Coimbatore (Fig. 1A).Actively growing shoots were used as the source of explants. The

shoots were washed under running tap water for 15 min. The explants were cut (1-2 cm) separately and they were washed with Tween 20 detergent solution for 10 min. After, they were thoroughly washed under running tap water until the traces of Tween 20 was removed. Remaining steps of surface sterilization was carried out under aseptic conditions in laminar air flow chamber. The shoots were then subjected to 70% ethanol treatment for one min and again washed with sterilized double distilled water at least three to four times. After washing with sterilized double distilled water, surface sterilization was done with mercuric chloride (0.12%w/v $HgCl_2$) solution for 3 min and rinsed four to five times with sterilized double distilled water. Sterilized explants were inoculated on medium for shoot induction.

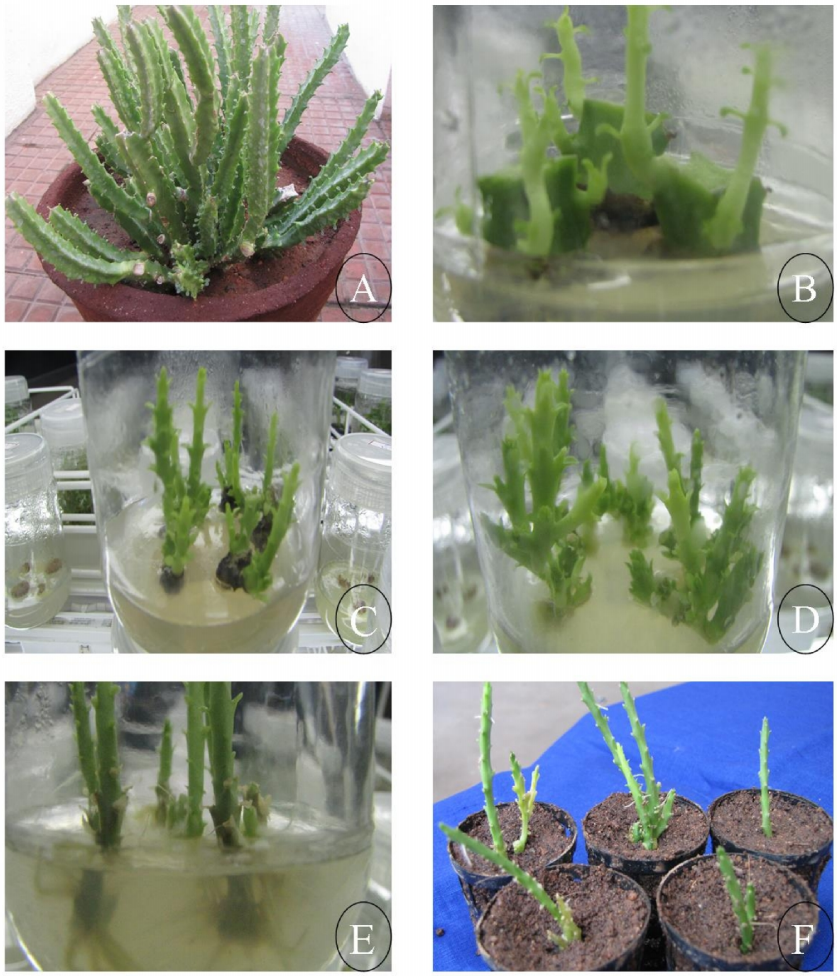

Fig. 1A. Habit B-Shoot bud differentiation from nodal explants on MS+ BAP (4.44 µM) + TDZ (0.90 µM) C&D-Different stage of multiple shoot formation E-Rooting of *in vitro* regenerated shoots on MS medium+ NAA (5.37µM)F-Hardened *in vitro* grown plant

2.2 Culture Media and Culture Conditions

A culture medium containing MS [24].Salts supplemented with macro elements, micro elements, 3% sucrose (HI Media, India) solidified with 0.8% (w/v) agar was used for the culture. The pH of the medium was adjusted to 5.8 by 1N NaOH or 1N HCL after adding the growth regulators prior to autoclaving. The media were steam sterilized in an autoclave under 15 psi and 121ºC for 20 min. All of the cultures were incubated under 50µ mol-2S-1 provided by cool white fluorescent lamp for a photo period of 16 h at 25±2ºC and 70-80% related humidity.

2.3 Shoot Initiation and Multiplication

For shoot induction, the nodal explants were cultured on MS medium supplemented with various plant growth regulators like BAP, TDZ, KIN and NAA either alone or in combinations for rapid shoot organogenesis (Table1). Twenty explants were used for each culture. The percent of explants responding for shoot formation were recorded after 35 days. In the subsequent sub cultures, the multiple shoots from the nodal explants were carried out at the regular interval of 15-20 days.

Table 1. Effect of BAP, TDZ, KIN and NAA, on initiation and multiple shoot induction from nodal explants of *C. diffusa* cultured on MS medium

S.NO	BAP µM	TDZ µM	KIN µM	NAA µM	Response %	Shoot No/explant	Shoot No/explants subculture	Shoot length cm
1	2.22	2.27	-	-	80.50	1.5±0.22	12.33±0.42	3.61±0.60
2	4.44	2.27	-	-	85.33	2.0±0.25	15.33±0.42	4.05±0.05
3	6.66	2.27	-	-	75.33	1.66±0.33	8.16±0.30	3.85±0.04
4	8.88	2.27	-	-	60.66	1.83±0.40	6.33±0.42	2.71±0.04
5	11.10	2.27	-	-	40.66	1.66±0.42	4.66±0.33	1.8±0.02
6	13.32	2.27	-	-	10.83	1.33±0.33	2.66±0.33	0.8±0.03
1	2.22	-	0.93	5.37	71.16	1.16±0.16	3.66±0.61	1.51±0.03
2	4.44	-	0.93	5.37	80.50	2.0±0.25	4.33±0.42	1.73±0.04
3	6.66	-	0.93	5.37	60.66	1.33±0.21	3.16±0.30	1.23±0.04
4	8.88	-	0.93	5.37	45.33	1.5±0.34	2.33±0.21	1.1±0.08
5	11.10	-	0.93	5.37	31.16	1.5±0.22	2.0±0.44	0.91±0.04
6	13.32	-	0.93	5.37	20.33	1.66±0.42	2.33±0.33	0.78±0.04
1	2.22	0.90	-	-	90.16	3.0±0.44	15.0±0.25	4.03±0.04
2	4.44	0.90	-	-	90.16	4.16±0.30	17.33±0.33	4.17±0.03
3	6.66	0.90	-	-	70.50	1.66±0.49	6.33±0.42	3.5±0.03
4	8.88	0.90	-	-	50.83	1.66±0.66	5.0±0.25	2.03±0.04
5	11.10	0.90	-	-	24.50	1.33±0.33	4.0±0.25	1.51±0.03
6	13.32	0.90	-	-	10.16	1.5±0.22	2.33±0.42	0.71±0.02
Basal medium	-	-	-	-	-	-	-	-

2.4 Rooting of *In vitro* Multiple Shoots

Shoots with 5-6 cm height were separated and individual shoots were transferred for rooting to MS medium containing different concentrations of NAA. The cultures were incubated under 16 h photoperiod for 15-20 days until the micro shoots developed the roots. Then the rooting frequency was measured (Table2).

Table 2. Effect of NAA on root formation of *C. diffusa* in MS medium

S.No.	MS medium +NAA µM	Response%	Mean number of roots/shoot	Mean root length (cm)
1	5.37	90.33	6.16±0.30	5.81±0.01
2	10.74	84.83	4.16±0.47	5.13±0.03
3	16.05	44.66	2.50±0.42	3.26±0.04
4	21.40	24.16	2.0±0.36	1.74±0.04
5	26.75	50.16	1.16±0.16	1.53±0.02
6	32.10	-	-	-
7	Basal medium	-	-	-

3. ACCLIMATIZATIONAND TRANSPLANTATIONOF PLANTLETS

The well-developed plantlets were removed from the culture bottles and washed with tap water to remove trace of agar and dipped in fungicide for few minutes. Then the plantlets were planted on to net pot contains different type of potting media (Table3) and survivability rate were determinate after 20 days of step-wise hardening processes. Hardened plants were transferred to pot containing mixture of decomposed coir waste, garden soil and vermiculite (1:1:1 ratio). The pots were watered at two days interval under shade house condition. After 60 days, the frequency of survival was calculated.

Table 3. Evaluation of different planting substrates for acclimatization of *In vitro* plantlets

S.No.	Planting substrates	No. of plants transferred	No. of plants survived	Survival (%)
1	Garden soil	50	28	55.83
2	Vermiculite	50	30	60.83
3	Decomposed coir waste	50	40	80.50
4	Hardening media-decomposed coir waste: garden soil: vermiculite	50	47	93.83

4. RESULTS AND DISCUSSION

The morphogenic response of nodal explants to BAP, TDZ, KIN and NAA are summarized in Table1. Placing explants in a medium without growth regulators (control) induced no shoots. However, the multiplication rate, shoot number was higher in culture supplemented with growth regulators. The percentage of response varied with varying concentrations of growth regulators used. All the concentrations of BAP facilitated shoot bud differentiation. Swelling of dormant axillary bud took place within eight days of inoculation and then differentiation into multiple shoots occurred after 35-40 days(Fig. 1B, C, &D). Of the various levels of BAP (2.22 to 13.32 µM) tested along with TDZ (0.90, 2.27 µM), KIN (0.93 µM) and NAA (5.37µM), the highest response percentage (90.16%), highest number of shoots per explants in initiation (4.16±0.30), with an average number of shoots per subculture (17.33±0.33) with a mean length of 4.17±0.03cm was recorded in MS medium fortified with BAP (4.44 µM) and TDZ (0.90 µM) (Table1). This is followed by MS+BAP (2.22 µM) and TDZ (0.90 µM) with response percentage (90.16%) number of shoots per explants in initiation (3.0±0.44) and an average number of shoots per subculture (15.0±0.25) with a mean length of 4.03±0.04. MS+BAP (4.44 µM) and TDZ (2.27 µM) with response percentage (85.33%) highest and number of shoots per explant in initiation (2.0±0.25), an average number of shoots per

subculture (15.33±0.42) and a mean length of (4.05±0.05 cm) (Table1). When compared to the above results the BAP, KIN and NAA combination is proved to be less effective. However, further increase in the concentration of BAP decreased the percentage of response, number of shoots in initiation, number of shoots per subculture and shoot length. Similar favorable combined effect of BAP and TDZ induced maximum number of shoots in subculture (7.0 ±0.19) was observed in *C. diffusa* [25]. However, in the present investigation the same growth regulators at lower concentration induces higher multiplication rate (17.33±0.33) during subculture when compared to the earlier report. All regenerated shoots were free from callus formation at their proximal end. The axillary buds present in the nodal segments have active meristem or quiescent depending upon the physiological state of the plant. These buds have the potential to develop into complete plantlets. The vegetative propagation of stem cutting *in vivo* relies on the auxiliary bud taking over the function of the main shoot in the absence of a terminal bud. In nature, these buds are rudimentary, remain dormant for a specific period depending on the growth pattern of the plant.

However, using micro propagation techniques the rate of shoot multiplication can be greatly enhanced by removing of apical bud, and performing auxiliary bud culture in a suitable nutrient medium containing appropriate cytokinin or cytokinin and auxin combination. Multiple shoot formation following the *in vitro* culture of nodal segments has proved to be an effective method of mass multiplication and commercially viable also. The effects of BAP on shoot initiation and multiple shoot formation has been demonstrated in a number of cases using a variety of explants [26]. In the present study, BAP along with TDZ also proved to be more effective. Supplement of TDZ at an optimal concentration (0.90 µM) had promotive influence on shoot development and multiple shoot formation. A high concentration of cytokinin along with the low concentration was most promoting for the induction and multiplication of shoots in *C. diffusa* and MS medium supplement with BAP (4.44 µM) in combination with TDZ (0.90 µM) proved most effective for direct shoot initiation and shoot proliferation also.

Response of multiple shoot proliferation was dependent on type and concentration of cytokinin supplements in the media. BAP along with TDZ shows the best result than compares with other combinations (Table1).Similarly, multiple shoot formation achieved through direct regeneration from the nodal explants of *B. diffusa* cultured on MS medium fortified with cytokinin [25]. In another report multiple shoot formation from nodal segments of *Ceropegia pusilla* occurred on the medium of MS+BAP (11.10µM) +NAA (5.37 µM) [27]. The efficiency of BAP in *in vitro* shooting has been successfully reported in other Asclepiadaceae species such as *Aristolochia bracteata* [28] and *Marsdenia brunoniana* [29]. The supplementation of BAP either singly or in combination with KIN for shooting was also reported in many plant species [30-32]. The shoots regenerated *in vitro* were transferred to MS medium supplemented with auxins. Root formation from the basal cut end of the shoots was observed seven days after transfer to the rooting medium without callus formation. The highest percentage (90.33%) of rooting was achieved in MS medium containing NAA (5.37 µM) (Fig. 1-E). The rooting frequency, number of roots per shoot and length of root were recorded after 20 days of culture. In most species, efficient rooting was observed on medium containing auxins. NAA and IBA are most commonly used plant growth regulators for root induction [33]. In the present study also NAA (5.37µM) induced the highest rooting frequency. The success of NAA is in promoting efficient root induction has been reported in *Boucerosiatruncato-coronata* [34].

5. ACCLIMATIZATION

Plant lets with well-developed roots were acclimatized inside the shade house with fogger system in selected planting substrate (Table 3). Partially hardened plantlets then transferred to the plastic pots containing red soil, sand and compost in the ratio of 1:1:1. Of the four different types of planting substrate examined, the percentage survival of the plantlets was highest (93.83) in hardening media (Table 3). During the hardening period, care was taken over the factors like temperature, light intensity, relative humidity, mineral nutrition and texture of the soil.

6. CONCLUSION

In conclusion, we have established a simple, reproducible, commercially viable direct *in vitro* culture system for an important medicinal plant *C. diffusa*. This can offers a potential system for conservation, improvement and mass multiplication of *C. diffusa* from node, thereby minimizing the pressure on wild population of the valuable flora of the forest. MS medium with BAP +TDZ (4.44 µM +0.90 µM) is the best for initiation and shoot multiplication. MS basal medium containing NAA (5.37µM) is the best for root induction. Among the various potting substrate used the hardening media was the best for maximum survival percentage (93.83%).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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