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An efficient *in vitro* protocol for mass multiplication of *Boucerosiatruncato-coronata* Sedgewick (Apocynaceae): a rare and an endangered medicinal plant

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ABSTRACT

An efficient and rapid method for *in vitro* propagation of *Boucerosiatruncato-coronata,*an endangered medicinal plant (Apocynaceae) was developed, resulting in shoot regeneration within 3 weeks of culture. Multiple shoots were regenerated from stem explants cultured on Murashige& Skoog (MS) medium containing 3% sucrose and supplemented with a range of BA (2.22-22.19µM), Kn (2.32-23.2µM) and IAA (0.57-2.85) concentrations. A 95% shoot response with a multiplication rate of five shoots per explant was obtained on MS medium containing 13.32µM BA, 4.65µM Kn and 0.57µM IAA. Callus produced at the base of the explant on the same medium showed root organogenic potential. The *in vitro* regenerated shoots produced roots when transferred to half strength MS medium with auxins. The micropropagated plants were easily acclimatized within 2 months under greenhouse conditions when potted in soil, sand and manure (1:1:1; v/v)mixture. More than 85% survival with no observable morphological variations was obtained. The developed protocol provides a simple, cost-effective means for the conservation of endangered *B. truncato-coronata* by clonal propagation within a short time.

Keywords : Conservation, Micropropagation, Succulent, endangered, Medicinal plant.

Genus *Boucerosia* (Subfamily: Asclepiadoideae, Family: Apocynaceae) was formerly included in the genus *Caralluma sensulato.* It is now segregated in to a separate genus *Boucerosiatruncato-coronata* Sedgewick. It is a succulent medicinal plant, destructively harvested by shepherds in Tumkur hill region of Karnataka. *Boucerosia*is found in dry regions of the world and reported to have significant anti-inflammatory and antitumor activity (Deepak *et al*. 1997, Zakaria *et al*. 2001). This has been attributed to thepregnane glycosides (Al-Harbi *et al*. 1994, Zakaria *et al*. 2002). The juicy stem of *B. tuberculata*is bitter tonic, febrifuge, stomachic and carminative useful in rheumatism and also consumed as vegetable (Shinwari *et al*. 2006). In another report, it was observed that*Caralluma fimbriata*, can be used in weight reduction (Lawrence & Choudhary 2004). *Borealluma edulis*is known for its anti-diabetic properties (Wadood *et al*. 1989) and other *Caralluma* species for their antihyperglycemic activity (Venkatesh *et al*.2003). 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. The indiscriminate and destructive harvesting of many of these plants continues unabated despite increased governmental regulation, resulting in many species (especially those with slow growth) becoming endangered (Nayar 1996). For the conservation of this medicinally important wild plant a simple, rapid and cost-effective protocol was developed for its clonal propagation.

MATERIAL & METHODS

Source material and bulking of explants– Stock plants collected along with roots from Pempanalli hills, Tumkur district, Karnataka during June 2007 were planted in pots (Figs. 1A, B) and maintained in green house atthe University Botanical garden. Based on our preliminary work, a standard decontamination procedure was followed. A quick wash of stem explants in 80% ethanol for 60 s was followed by soaking in distilled water containing a few drops of Tween 20 for 20 m. and washing thoroughly in running tap water. The explants were then sterilized in 0.1% (w/v) mercuric

chloride solution for 10 min. Finally the explants were rinsed five times with sterile distilled water. The surface decontaminated stem explants were cut into 10 mm lengths and then inoculated on 10 ml (in culture tubes, 100 mm x 25 mm, 40 ml volume) of full strength MS medium (Murashige & Skoog 1962) supplemented with 30 g l^{-1} sucrose, 0.1 g l^{-1}myo-inositol, 6-benzylaminopurine 2.22 – 22.19 µM (BA), 2.32 – 23.2 µM kinetin (Kn), 0.57-2.85 µM Indole 3-acetic acid (IAA) and solidified with 0.8% agar (Bacteriological agar Himedia, Mumbai, India). The pH of the medium was adjusted to 5.6 – 5.8 with 1N NaOH or 1N HCl before autoclaving at 121ºC at 15 psi for 20 min. Cultures were incubated in a growth room under a photoperiod of 16 h light provided by cool white fluorescent tubes (30 µMol $m^{-2}s^{-1}$) at 25±ºC.

Shoot multiplication–Shoot multiplication experiments were designed using nodal explants, excluding terminal portions, and cut into 10 mm lengths. The nodal cuttings were cultured on MS medium supplemented with BA (2.22-22.19 µM) and Kn (2.32-23.2 µM) alone or in combination with IAA (0.57-2.85 µM). Each treatment had 15 replicates and the experiment was repeated thrice. Cultures were incubated under the same growth conditions stated above. After 9 weeks, the total number of axillary shoots per explant (shoot multiplication rate) and length of shoots were recorded.

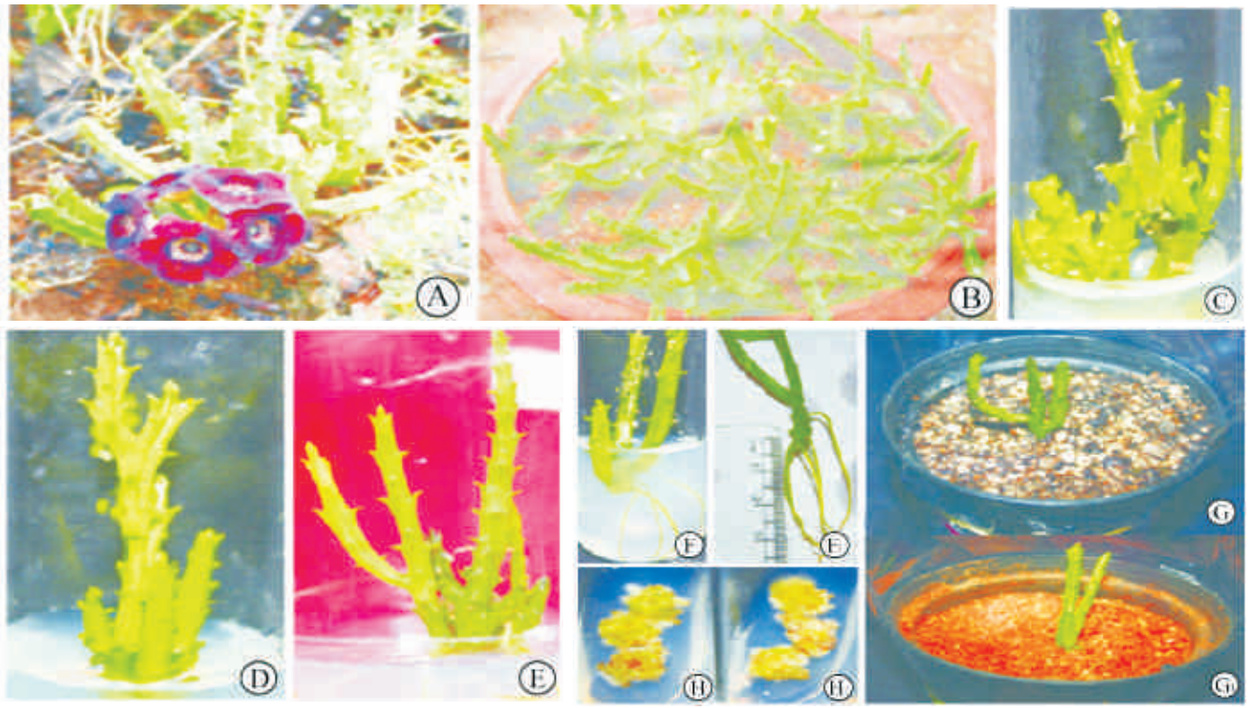
Rooting and acclimatization–For rooting of individual shoots, shoot clusters produced in the shoot multiplication stage were carefully separated and cultured in plant growth regulators (PGR)-free half-strength MS medium as well as half-strength MS medium supplemented with different concentrations of Naphthalene acetic acid (NAA) (0.54, 1.07, 1.61, 2.15, 2.69, and 5.37µM), IAA (0.57, 1.14, 1.71, 2.28, 2.85, and 5.71µM) and Indole butyric acid (IBA) (0.49, 0.98, 1.48, 1.97, 2.46 and 4.9 µM) for 60 days. Independent rooted shoots, produced from nodal explants were gently extracted from the vessels and washed thoroughly with tap water to remove adhered agar and traces of the medium. The plantlets were given a final wash in distilled water for 5 min and transferred to plastic cups (8cm in diameter) containing soilrite. The pots were maintained in a polythene cover in the culture room and irrigated once a week with half strength MS liquid medium. Polythene covers were removed gradually, and the plants were transferred to earthen pots containing garden soil for further growth in greenhouse.

Callus study– Callus produced at the base of stem explantsandwas sub-cultured on the same medium to produce more calli. A completely randomized experiment was designed to determine the organogenic potential of thiscalli. Concentrations of 0.00, 0.57, 2.69 and 5.37µM NAA were combined in a factorial manner with 0.00, 2.22, 4.44 and 8.87µM BA. Cultures were incubated in a growth room under a photoperiod of 16 h light provided by cool white florescent tubes (30 µMol$m^{-2}s^{-1}$) at 25 ± 2ºC. Each treatment had 15 replicates. After 6 weeks, presence or absence of organogenesis was recordedand callus fresh weight recorded.

Data analysis–Mean values of the various treatments were subjected to analysis of variance using SPSS version 1.5. Significance level was determined at *P*=0.05. Where there were significant differences, the means were separated using Duncan's Multiple Range Test (Gomez & Gomez 1976).

RESULTS & DISCUSSION

Shoot regeneration : Multipleshoots were induced from axillary buds. Nodal cuttings were cultured on MS medium supplemented with BA and Kn alone or in combination with IAA (Figs. 1 C, D & E). The axillary bud proliferation ability of the explant varied depending on the plant growth regulators and concentrations used. Shoots formed with all of the combinations was investigated. However, there were no shoots on MS basal medium (control). Bud break usually occurred after one week of culture. The percentage of response, number of shoots and average shoot length were controlled by the type and concentration of the growth regulators employed. On MS medium supplemented with Kn (2.32-23.2 µM), the number of shoots was limited to two or three (Table1). A maximum of 60% of the cultures produced shoots, with an average number of 2.4 shoots per explant, when 4.65 µM Kn was used. The average length of the shoot was limited to 3.06 cm after 60 days of culture on this medium (Fig. 1D). However, increase or decrease in the concentration of Kn, resulted in the decrease in the number and length of the shoots.



Fig—1. *In vitro* propagation of *Boucerosiatruncato-coronata*.A. Natural habit with flower, B. Garden plant, C. Shoot regeneration from the axillary bud of nodal cuttings on MS medium containing 13.32 µM BA, D. Multiple shoot induction from medium containing 4.65 µM Kn, E. Multiple shoot production on MS medium supplemented with 13.32 µM BA, 4.65 µM Kn and 0.57 µM IAA,F & F. Rooted shoot just before transfer to soil. The Shoots were extracted from half-strength MS medium supplemented with 2.85 µM IAA, G. Acclimatized plants growing in plastic cups 45 days after transfer to soil and H. Green callus growth with root hairs on MS medium supplemented with 5.37 µM NAA and 8.87 µM BA.

Another cytokinin, BA, was added to the MS medium at concentrations of 2.22-22.19 µM to investigate whether it plays any critical role in multiple shoot induction (Table 1). BA was better than Kn in terms of number of shoots produced per explant as well as the percentage of the cultures that responded. On MS medium supplemented with 13.31 µM BA, 80% of the cultures responded with an average number of 4.6 shoots per explant with average shoot length of 3.23 (Fig. 1C). However, the average shoot length decreased compared to shoots developed on medium containing Kn alone. The addition of IAA (0.57 and 2.85 µM) along with 13.32 µM BA and 4.65 µM Kn increased the shoot length significantly. However the average number of shoots showed a slight decrease compared to 13.31 µM BA alone. On MS medium supplemented with 13.32 µM BA, 4.65 µM Kn and 0.57 µM IAA, 95% of the cultures responded with an average number of 4.60 shoots with an average shoot length of 4.83cm (Fig. 1E; Table 1). Hence the addition of IAA enhanced the average shoot length. Shoot length plays a crucial role in the rooting of shoots in this system.

Multiple shoot induction was achieved using various concentrations of Kn and BA alone or in combination with IAA. Addition of IAA to medium considerably enhanced multiple shoot induction. From the published reports on various systems, it is clear that cytokinin is essential for multiple shoot induction from nodal cuttings. BAinduced multiple shootinduction has been reported in *Marsdenia brunoniana* (Ugraiah *et al*. 2010) and *Sarcostemma brevistigma* (Thomas & Shankar 2009). However, the poor performance of Kn in present study is contradictory to the reports on asclepiads such as *Ceropegia spiralis* (Murthy *et al.* 2010), and *Carallum aedulis* (Rathore *et al*. 2008). However, the individual use of cytokinins was not found

Table 1— Effect of BA and Kn alone and in combination with IAA on adventitious multiplication of nodal explants of *B. truncato-coronata* 60 days.

Plant growth regulators (µM)			Shoot sprouting frequency (%) per explant [a] ± SE	Mean shoot number ± SE	Mean length of shoots[a] (cm)	Basal callusing
BA	Kn	IAA				
2.22	-	-	66	2.23 ± 0.18 [d]	3.06± 0.24 [bc]	-
4.44	-	-	71	2.83 ± 0.09 [c]	2.56 ± 0.12 [de]	-
8.87	-	-	75	3.30 ± 0.24 [b]	4.83 ± 0.12 [a]	-
13.31	-	-	80	4.60 ± 0.08 [a]	3.23 ± 0.17 [b]	-
22.19	-	-	68	2.26 ± 0.09 [d]	2.50 ± 0.14 [d]	-
-	2.32	-	46	2.26 ± 0.18 [b]	2.90 ± 0.24 [b]	-
-	4.65	-	60	2.40 ± 0.08 [a]	3.06 ± 0.20 [a]	-
-	9.29	-	47	2.30 ± 0.08 [b]	2.80 ± 0.08 [d]	-
-	13.94	-	37	2.03 ± 0.04 [bc]	2.93 ± 0.18 [bc]	-
-	23.2	-	20	1.90 ± 0.03 [d]	2.90 ± 0.21 [cd]	-
13.31	4.65	0.57	95	5.26 ± 0.33 [a]	3.36 ± 0.12 [a]	-
13.31	4.65	2.85	91	3.60 ± 0.08 [b]	2.80 ± 0.14 [ab]	++

Medium MS [a] The values represents the means (± SE) of three independent experiments. At least 24 cultures were raised for each experiment. Mean values with in a column followed by the same letter are not significantly different by Duncan's multiple range test ($P > 0.05$). - sign indicates no callusing No. of + signs represents the intensity of callusing.

to give optimum results in most of the systems; the best result was obtained when Kn and BA was employed in combination with an auxin. Multiple shoot induction using an auxin-cytokinin combination is a common protocol, as it has been reported for several systems (Sudha *et al*. 2000, Martin 2002). The synergistic effect of BA and Kn in combination with IAA has been reported for some Asclepiadoideae medicinal plants such as *Caralluma edulis* (Rathore *et al*. 2008) and *Ceropegia juncea* (Nikam & Savant 2009).

In *Achrass apota*, the induction of multiple shoots during culture initiation was found to be usually low when BA was employed for shoot proliferation. However, in subsequent subcultures the number of shoots increased by up to three-fold (Purohit & Singhvi 1998). On the other hand, we obtained the maximum number of multiple shoots during the culture initiation step in the present study, and subsequent subcultures of the shoots did not yield higher number of shoots. However this single step procedure can save time, since subsequent subcultures are not necessary to achieve optimum results.

Rooting and acclimatization–Individual shoots, at least 2-3 cm long, were separated and cultured on half strength MS medium supplemented with NAA (0.54, 1.07, 1.61, 2.15, 2.69, and 5.37 µM), IAA (0.57, 1.14, 1.71, 2.28, 2.85 and 5.71 µM) and IBA (0.49, 0.98, 1.48, 1.97, 2.46 and 4.9 µM) for root induction. The shoots that were below 1.0 cm in length did not produce any roots when cultured on rooting medium (data not shown). The shoots produced roots at all concentration of IAA, NAA and IBA, but the highest efficiency of 88% rooting was observed in 2.85 µM IAA. The development of an average number of 4.6 roots from a single shoot was noticed in 2.85µM IAA fortified medium at the end of the 60th day of culture (Table 2; Figs. 1F & 1F$_1$). The roots were formed at the basal ends of the shoots. IAA was the most effective auxin in terms of percent shoots forming roots and root length and has been reported in *Ceropegia juncea* (Nikam & Savant 2009). IBA favoured rooting in other Asclepiad medicinal plants such as *Tylophoraindica* (Faisal & Anis 2005), *Sarcostemmabrevistigma* (Thomas & Shankar 2009), *Huerniahystrix* (Amoo *et al*. 2009),

Table 2— Effect of different auxins on rooting of in vitro developed shoots in *B. truncato-coronata*

Plant growth regulators (mg/l)			% of response	Mean number of roots per shoots ± SE	Mean length of roots (cm) ± SE	Degree of callusing
NAA	IAA	IBA				
0.54	-	-	60	1.73 ± 0.12 ^{f}	8.10 ± 0.16 ^{a}	-
1.07	-	-	60	3.43 ± 0.04 ^{a}	2.33 ± 0.09 ^{de}	-
1.61	-	-	55	1.83 ± 0.04 ^{e}	2.46 ± 0.12 ^{cd}	-
2.15	-	-	40	1.93 ± 0.12 ^{d}	2.23 ± 0.12 ^{ce}	+
2.69	-	-	66	3.16 ± 0.17 ^{b}	2.66 ± 0.18 ^{c}	-
5.37	-	-	63	2.20 ± 0.00 ^{c}	4.20 ± 0.24 ^{b}	++
-	0.57	-	55	3.70 ± 0.08 ^{c}	1.03 ± 0.12 ^{e}	-
-	1.14	-	40	1.16 ± 0.04 ^{e}	1.73 ± 0.17 ^{a}	-
-	1. 71	-	66	4.43 ± 0.04 ^{b}	1.33 ± 0.04 ^{bcd}	-
-	2.28	-	60	4.03 ± 0.09 ^{c}	1.46 ± 0.12 ^{bc}	-
-	2.85	-	88	4.60 ± 0.14 ^{a}	1.73 ±0.04 ^{a}	-
-	5.71	-	71	1.26 ± 0.04 ^{e}	1.56 ± 0.18 ^{b}	++
-	-	0.49	-	**CP**	**CP**	+
-	-	0.98	-	**CP**	**CP**	++
-	-	1.48	-	**CP**	**CP**	++
-	-	1.97	-	**CP**	**CP**	+++
-	-	2.46	55	2.10 ± 0.08 ^{a}	1.43 ± 0.04 ^{b}	-
-	-	4.90	50	1.16 ± 0.09 ^{b}	1.66 ± 0.17 ^{a}	-

Means ± SE, n=45. Means followed by the same letter are not significantly different by the DMR test at 0.05% probability level; - sign indicates no callusing, No. of + signs represents the intensity of callusing **CP** – Callus production.

Table 3— Effect of combinations of NAA and BA concentrations on callus growth

PGR combination NAA: BA (µM)	Mean callus fresh weight (g)
Control	00.00
0.00:2.22	0.14 ± 0.009^{f}
0.00:4.44	0.17 ± 0.134^{f}
0.00:8.87	0.20 ± 0.020^{f}
0.54:0.00	0.26 ± 0.024^{f}
0.54:2.22	0.55 ± 0.048^{e}
0.54:4.44	0.89 ± 0.080^{d}
0.54:8.87	0.71 ± 0.073^{dc}
2.69:0.00	0.85 ± 0.051^{d}
2.69:2.22	1.36 ± 0.100^{c}
2.69:4.44	1.81 ± 0.092^{b}
2.69:8.87	1.90 ± 0.147^{b}
5.37:0.00	0.66 ± 0.047^{de}
5.37:2.22	1.18 ± 0.102^{c}
5.37:4.44	1.89 ± 0.096^{b}
5.37:8.87	2.42 ± 0.189^{a}

Mean values followed by the same letter(s) are not significantly different ($P = 0.05$) based on DMRT

Caralluma bhupenderiana (Ugraiah *et al.* 2011), *Boucerosia diffusa* (Ramadevi *et al.* 2012) *Ceropegia candelabrum* (Beena *et al*. 2003), *Holostemaada-hodein* (Martin 2002) and *Gymnema sylvestre* (Komalavalli & Rao 2000).

The rooted shoots were successfully transplanted to plastic cups containing soilrite and the humidity was maintained at approximately 90% by covering with polythene bag. The plants were irrigated once a week with a solution of half strength MS medium. After two months the plants were transferred to larger pots containing garden soil, and after acclimatization, 50 plants transferred to soil, 43 survived with a survival frequency of 86% (Fig. 1G). Plants transferred to the field established themselves in soil and are growing well.

Callus study– The effects of different combinations of BA and NAA on callus growth are presented in Table 3. Generally, callus fresh weight increased (significantly in many cases) with increased BA concentration at the same NAA concentration. Similarly, there was a significant increase in most cases with increased NAA concentration at the same level of BA concentration. This suggests that both NAA and BA have synergistic effects on callus growth in *B. truncato-caronata.* Thomas (2009) reported shoot organogenesis from callus developed from mesophyll protoplasts of *Tylophoraindica* on medium fortified with thidiazuron (TDZ) (1-7 µM) and naphthalene acetic acid (NAA) (0.2-0.4 µM). On the other hand, Patnaik &Debata (1996) reported no shoot formation from *Hemidesmusindicus* callus. However, in the present study, root hair like structures observed in some cases (Fig. 1H) demonstrates the organogenic capacity of the induced callus. There may be a need to test other growth regulators for shoot induction from the derived callus and evaluate the possibility of inducing somatic embryos.

The successful micropropagation system described here provides an effective means for the conservation and rapid clonal propagation, within a short time, of endangered medicinal plant *B. truncato-coronata.* The increased multiplication rate and cost effective, easy acclimatization process make this protocol highly advantageous. Moreover, the application of this developed protocol to some other succulent species in our laboratory shows promising results.

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