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IN VITRO PROPAGATION STUDIES OF A THREATENED MEDICINAL PLANT *BOUCEROSIA INDICA* WT. & ARN.

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The present work is intended to establish a reproducible protocol for rapid production of multiple shoots, callus cultures, rooting of *in vitro* regenerated shoots, hardening and transfer to the field conditions of a threatened medicinal plant *Boucerosia indica*. Nodal explants on Murashige and Skoog (MS) medium supplemented with combination of Benzyl-6-aminopurine 2 mg/l and 2-Isopentyl adenine 1 mg/l showed very high percentage of shoot sprouting frequency. Internodal explants on MS medium with 2,4-Dichlorophenoxy acetic acid 2.0 mg/l showed maximum response of callus proliferation. Subculture of above calli induced with Benzyl-6-aminopurine 2.0 mg/l was best for organogenesis. *In vitro* raised shoots rooted most excellent on ½ strength MS medium with Naphthalene-3-acetic acid 0.1 mg/l and those i*n vitro* rooted plants showed 85% survival rate all through acclimatization.

Key words: *In vitro* propagation, *Boucerosia indica,* Plant tissue culture, Explants, Regeneration

In vitro regeneration is an efficient means of ex situ conservation of plant diversity (Krogstrup *et al*. 1992; Fay 1994). Furthermore this technique has the unique advantage of propagation of the desired taxon, independent of season, reproductive barriers, germination problems and so on (Anuradha and Pullaiah 2001). The family Asclepiadaceae comprises more than 348 genera and about 2900 species, these are chiefly located in the tropics and subtropics. *Boucerosia indica* Wt. & Arn. belongs to Asclepiadaceae according to the classical systems of classification, but according to APG III system, it is now placed under the sub family Asclepiadoideae of Apocynaceae. The genus *Boucerosia* is separated from the genus *Caralluma* (*sensu stricto*) mainly by the psudoumbellate terminal cymes (Plowes 1995). Genus *Boucerosia* comprises eight species and these are endemic to Peninsular India and Sri Lanka. *Boucerosia indica* is one of the lesser known stapeliads in southern India. *Boucerosia indica* is locally recognized as *Elika chevulu*, *Elukam chevulu* and *Kundeti kommulu* and distributed in Andhra Pradesh, Tamilnadu and Karnataka.

Plants belonging to this genus are rich in esterified polyhydroxy pregnane glycosides. The genus is also characterized by the presence of flavone glycosides (Ramesh *et al*. 1999b, Rizwani *et al*. 1990). The species of *Boucerosia* and *Caralluma* found in India are edible and the medicinal properties of *Boucerosia* and *Caralluma* include anti-inflammatory, anti-nociceptive, anti-ulcer, anti-diabetic, anti-pyretic and anti-oxidant effects. *Boucerosia* and *Caralluma* extracts have also been found to be appetite suppressant, a property which is well known to Indian tribals.

MATERIALS AND METHODS

Boucerosia indica plants along with its roots were collected from Bakarapet foot hills of Chittoor district of A.P., India. The plants were potted and maintained in the S. K. University botanical garden, Anantapur, Andhra Pradesh, India. The plants were grown at 30° - 40°C with natural daylight and irrigated with water as required. After 4 weeks young shoots that grew were used as the explants source for *in vitro* propagation studies. Aggressively growing shoots with five to seven nodes were the source of explants. The explants material was immersed in 70% alcohol for 1 min and again washed with sterilized double distilled water. Later surface sterilization was done with sterilants like $HgCl_2$ (0.05% to 1.0%) NaOCl (5% to 30%) and H_2O_2 (10% to 30%)

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maintaining various time intervals (1, 2, 5, 6, 8 and 10 min). After treatment with sterilant, the explants were thoroughly rinsed with several changes of sterile double distilled water. Then those explants were inoculated on to culture medium vertically to maintain polarity. All cultures were maintained at 25 $\pm 2^0$C with 16/8 h photoperiod under flourescent tubes with a light intensity of 2000 lux.

Surface sterilized juvenile explants such as node, internodes and shoot tip explants were cut into suitable sizes and cultured on MS medium supplemented with 3% sucrose and different concentrations of BAP, Kn, 2-iP etc. After selecting the best explants for shoot regeneration, it was cultured on three different agarified media i.e. MS, B5 and WPM supplemented with 2 mg/l BAP and 3% sucrose. After selecting the best explants and medium for shoot multiplication further experimental manipulations were carried to assess the morphogenic influence of different PGRs such as auxins (1AA, NAA, 1BA, 2,4-D, 2,4,5-T and Picloram), cytokinins (BAP, Kn, 2-iP) and other organic supplements and different sugars (glucose, fructose, sucrose, galactose and maltose). Stem segments were cultured on three different media such as MS, B5 and WPM supplemented with 2 mg/l 2,4-D were tested for callus induction and regeneration of plantlets. Stem segments were cultured on selected media supplemented with different PGRs such as 2,4-D, 2,4,5-T, Picloram, NAA, IAA, IBA, Kn and in which combination the best callus production was observed. Callus regeneration or organogenic callus was sub-cultured on basal medium supplemented with different concentrations of cytokinins and combinations for shoot development.

For *in vitro* rooting of individual regenerated shoots with 4 - 6 nodes were cultured on different media such as full strength and half strength MS, B5 and WPM media supplemented with different concentrations of IAA, IBA and NAA, 1% sucrose and 0.6% agar. *In vitro* raised plantlets were planted in earthen ware pots containing sterilized sand, farm yard manure and peat moss in 1:1:1 ratio covered with polythene bags and incubated at 24 $\pm 2^0$C for 20 days. Slowly the pots were transferred to room temperature having diffused light and plants were shifted to the green house then finally plants were transferred to the field by directly exposing to the sunlight. The data was statistically analyzed using one-way analysis of variance (ANOVA) and means were compared using the DMR test at the 0.05% level of significance.

RESULTS AND DISCUSSION

Surface sterilization of *Boucerosia indica* was carried out with different sterilants/

Table1: Effect of various combinations of BAP, 2-iP and Kn on shoot formation from nodal explants of *Boucerosia indica* on MS medium

Plant growth regulators (mg/l)			Shoot sprouting frequency (%)	Mean number of shoots per explant ± SE	Mean length of shoots (cm) ± SE
BAP	2-iP	Kn			
1	0.5	-	53	1.32 ± 0.14 ^{dc}	1.30 ± 0.12 ^{df}
1	1.0	-	73	1.96 ± 0.06 ^{d}	2.50 ± 0.30 ^{g}
1	2.0	-	66	1.45 ± 0.09 ^{e}	1.35 ± 0.08 ^{b}
1	3.0	-	60	1.04 ± 0.16 ^{b}	1.15 ± 0.06 ^{bc}
1	-	0.5	46	1.46 ± 0.13 ^{d}	1.28 ± 0.18 ^{fh}
1	-	1.0	66	1.54 ± 0.20 ^{e}	1.51 ± 0.16 ^{g}
1	-	2.0	60	1.32 ± 0.05 ^{dcf}	1.22 ± 0.15 ^{be}
1	-	3.0	53	1.16 ± 0.12 ^{a}	1.20 ± 0.12 ^{b}
2	0.5	-	66	2.14 ± 0.32 ^{dc}	1.45 ± 0.22 ^{a}
2	1.0	-	88	4.91 ± 0.30 ^{h}	5.13 ± 0.14 ^{g}
2	2.0	-	73	1.50 ± 0.22 ^{e}	1.33 ± 0.06 ^{g}
2	3.0	-	66	1.49 ± 0.19 ^{d}	1.25 ± 0.12 ^{bd}
2	-	0.5	53	1.40 ± 0.04 ^{abc}	1.36 ± 0.08 ^{bc}
2	-	1.0	66	1.73 ± 0.21 ^{b}	2.44 ± 0.17 ^{bcd}
2	-	2.0	60	1.66 ± 0.06 ^{bc}	1.50 ± 0.20 ^{a}
2	-	3.0	53	1.20 ± 0.08 ^{f}	1.26 ± 0.21 ^{abc}
3	0.5	-	66	1.47 ± 0.00 ^{bd}	1.28 ± 0.22 ^{f}
3	1.0	-	86	2.50 ± 0.07 ^{b}	1.80 ± 0.16 ^{ef}
3	2.0	-	73	1.79 ± 0.08 ^{bc}	1.46 ± 0.18 ^{de}
3	3.0	-	60	1.35 ± 0.13 ^{a}	1.25 ± 0.04 ^{a}
3	-	0.5	53	1.16 ± 0.14 ^{a}	1.28 ± 0.09 ^{c}
3	-	1.0	66	1.67 ± 0.05 ^{b}	1.50 ± 0.22 ^{ab}
3	-	2.0	53	1.44 ± 0.14 ^{bc}	1.20 ± 0.24 ^{a}
3	-	3.0	46	1.10 ± 0.06 ^{bc}	1.16 ± 0.10 ^{bcd}
-	1	0.5	53	1.36 ± 0.16 ^{b}	1.50 ± 0.08 ^{f}
-	1	1.0	66	1.58 ± 0.01 ^{ce}	1.72 ± 0.21 ^{bc}
-	1	2.0	46	1.28 ± 0.12 ^{c}	1.45 ± 0.04 ^{b}
2	1	0.5	53	1.62 ± 0.13 ^{b}	1.57 ± 0.14 ^{c}
2	1	1	66	1.81 ± 0.05 ^{a}	2.00 ± 0.33 ^{a}

Values represent Means + (Standard error) SE, n = 45.
Means followed by the same letter are not significantly different by the DMR test at 0.05% probability level.

Table 2: Influence of various Auxins on callus induction from internodal explants of *Boucerosia indica* cultured on MS medium

Plant growth regulators (mg/l)	Frequency of response (%)	Degree of callusing	Nature of callus
2,4-D			
0.1	NR	NR	NR
0.5	40	+	PGFC
1.0	46	+ +	PGFC
2.0	87	+ + +	GCC
3.0	73	+ + + +	PGFC
4.0	66	+ + +	YFC
5.0	53	+ +	CFC
2,4,5-T			
0.1	53	+ +	LGEC
0.5	60	+ + +	LGCC
1.0	73	+ + + – –	PGCC
2.0	66	+ + + +	PGFC
3.0	60	+ + +	WCC
4.0	53	+ +	BPC
5.0	46	+	BFC
Picloram			
0.1	NR	NR	NR
0.5	NR	NR	NR
1.0	53	+	LGPC
2.0	73	+ + + +	GFC
3.0	66	+ + +	LGPC
4.0	53	+ +	GFC
5.0	40	+	YFC

BFC- Brown Friable Callus
BPC- Brown Profuse Callus
CFC- Creamish Friable Callus
GCC- Green Compact Callus
GFC- Green Friable Callus
LGEC- Light green Embryogenic Callus
LGFC- Light green Friable Callus
LGPC- Light green Profused Callus
PGCC- Pale Green Compact Callus
PGFC- Pale Green Friable Callus
WCC- Whitish Compact Callus
YFC- Yellowish Friable Callus
+ - Very Less
+ + - Less
+ + + - Moderate
+ + + + - Above Moderate
+ + + + + - Good Response
NR No response

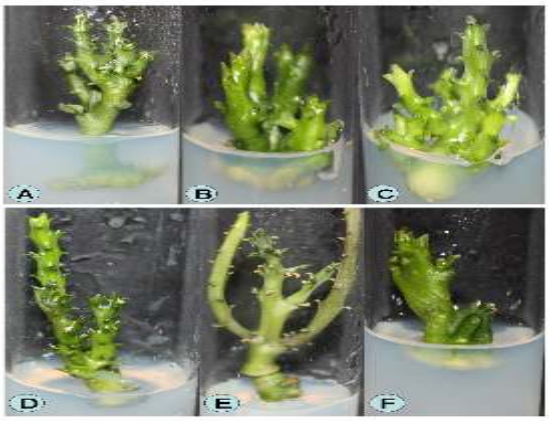

Fig. 1 Effect of various Cytokinin combinations on Multiple shoot induction and multiplication of *Boucerosia* indica. (A) MS + BAP 1.0 mg/l + 2-iP 1.0 mg/l; (B) MS + BAP 2.0 mg/l + 2-iP 1.0 mg/l; (C) MS + BAP 3.0 mg/l + 2-iP 1.0 mg/l; (D) MS + BAP 2.0 mg/l + Kn 1.0 mg/l; (E) MS + 2-iP 1.0 mg/l + Kn 1.0 mg/l; (F) MS + BAP 2.0 mg/l + 2-iP 1.0 mg/l + Kn 1.0 mg/l

Table 3: Effect of various Auxins on rooting response from *in vitro* regenerated shoots of *Boucerovia indica* cultured on MS half strength medium

Different concentrations of Auxins			% of response	Mean number of roots per explant ± SE	Mean length of roots (cm) ± SE
IAA	IBA	NAA			
0.1	-	-	53	5.25 ± 0.14 [c]	1.50 ± 0.16 [b]
0.5	-	-	66	7.12 ± 0.06 [a]	2.48 ± 0.10 [ab]
1.0	-	-	60	3.25 ± 0.04 [d]	1.40 ± 0.14 [b]
2.0	-	-	46	2.16 ± 0.12 [e]	1.22 ± 0.18 [b]
3.0	-	-	NR	NR	NR
	0.1	-	46	3.19 ± 0.17 [d]	2.24 ± 0.21 [ab]
-	0.5	-	66	5.28 ± 0.02 [c]	3.09 ± 0.06 [a]
-	1.0	-	60	2.19 ± 0.08 [e]	1.34 ± 0.17 [b]
-	2.0	-	NR	NR	NR
-	3.0	-	NR	NR	NR
-	-	0.1	75	9.57 ± 0.05 [a]	3.21 ± 0.12 [ab]
-	-	0.5	66	6.24 ± 0.09 [b]	3.12 ± 0.14 [b]
-	-	1.0	60	4.19 ± 0.06 [d]	1.42 ± 0.22 [b]
-	-	2.0	NR	NR	NR
-	-	3.0	NR	NR	NR

Values represent Means ± (Standard error) SE, n 45. Means followed by the same letter are not significantly different by the DMR test at 0.05% probability level.

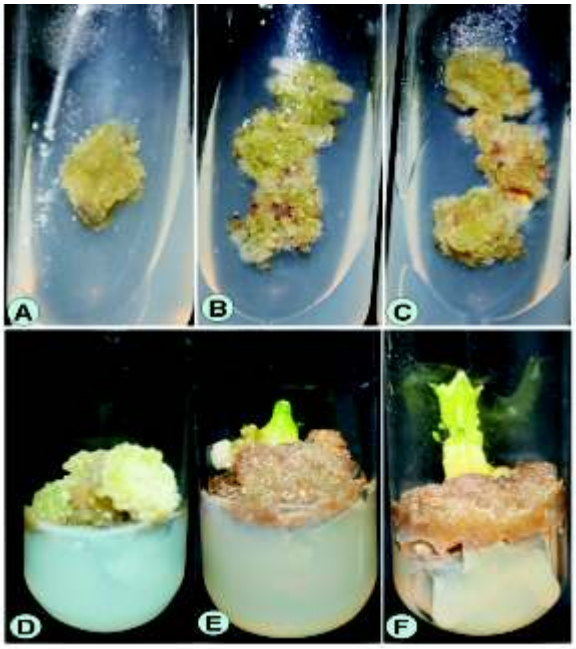

Fig. 2 Effect of various Auxins and Cytokinins on Callus culture and organogenesis studies of *Boucerosia* indica (A) MS + 2,4,5-T 1.0 mg/l; (B) MS + Picloram 2.0 mg/l; (C) MS + 2,4-D 2.0 mg/l; (D) MS + Kn 1.0 mg/l; (E) MS + 2-iP 2.0 mg/l; (F) MS + BAP 2.0 mg/l

Fig. 3: Effect of various Auxins on *In vitro* rooting and acclimatization of *Boucerosia indica* (A,B) ½ MS + NAA 0.5 mg/l; (C,D) ½ MS + NAA 0.1 mg/l; (E,F) *In vitro* rooted plants transferred to soil rite in plastic pots and covered with polythene bags; (G,H) Acclimatized *in vitro* grown plants

disinfectants like mercuric chloride ($HgCl_2$), sodium hypochloride (NaOCl) and hydrogen peroxide (H_2O_2). Both the concentration and duration of exposure to disinfectants are essential. If they are respectively too high or too long, the plant tissues will be damaged, but too mild an exposure will not destroy the microorganisms. Out of various surface sterilants ($HgCl_2$, NaOCl and H_2O_2) tested for raising aseptic cultures, $HgCl_2$ 0.1% for about 3 minutes duration was found to be effective (80%) in obtaining healthy shoot proliferation without contamination. Prior to this, other *Carallumas* established with surface sterilization of 0.1% mercuric choloride include *Caralluma pauciflora* (Kiranmai 2006), *Caralluma edulis* (Rathore *et al.* 2008), *Caralluma sarkariae* (Sreelatha *et al.* 2009) and three varieties of *Caralluma adscendens* (Aruna *et al*. 2009).

Multiple shoot induction and multiplication

The morphogenic response of explants depends on type of explants and physiological status. In general juvenile tissue shows more response than mature tissue. Choice of the correct explants is imperative when propagation is to be based on direct or indirect shoot initiation. For selecting best explants capable of active proliferation of multiple shoots, different explants like node, inter node and shoot tip of *Boucerosia indica* were cultured on MS, B5 and WPM media supplemented with 3% sucrose. Nodal explants responded with bud break and emergence of bud within 1 - 2 weeks on MS medium. Multiple shoot induction was maximum (86%) from nodal explants on MS medium fortified with BAP 2.0 mg/l with 3.09 ± 0.23 shoots per explant and maximum length of shoots 3.00 ± 0.13 cm than Kn, and 2-iP when used individually in this species. In the present investigation on *Boucerosia indica* nodal segments were found to be more responsive than shoot tip explants for multiple shoot production. Node culture is of value for propagating species that produce elongated shoots in culture. There are numerous examples to show that nodal explants are most effective in Asclepiadaceae, these include; *Caralluma edulis* (Rathore *et al.* 2008) and three varieties of *Caralluma adscendens* (Aruna *et al*. 2009).

Composition of the medium plays a foremost role in enhancing shoot proliferation. To develop efficient plant regeneration protocol for *in vitro* multiplication techniques, evaluation of medium is a prerequisite step. For shoot proliferation from *Boucerosia indica* nodal segments were cultured on different basal media at full strength such as MS medium with high salt concentration. B5 medium with medium salt concentration and WPM with low salt concentration with addition of BAP 2.0 mg/l and 3% sucrose. In the present investigation induction of axillary buds was observed on three media, but percentage of

response, number of shoots per explant and average shoot length were more in MS medium followed by B5 and WPM. Nodal explants of *Boucerosia indica* produced 2.09 ± 0.13 shoots per explant with 2.00 ± 0.17 cm length on MS medium and better regeneration (82%) on MS medium.

Growth regulators play a key role in influencing the morphogenic potentiality in tissue culture systems. Cytokinins can promote the maturation of chloroplasts and delay the senescence of detached leaves. For high frequency regeneration of multiple shoots, a range of cytokinins either individually or in combination with other cytokinins were used. Sometimes addition of more than one cytokinin to the medium results in enhanced shoot production or shoots of superior quality. When cytokinin combinations were tested for shoot multiplication from nodal explants of *Boucerosia indica*, BAP (2.0 mg/l) + 2-iP (1.0 mg/l) was found to be efficient (88%) with maximum number of 4.91 ± 0.30 shoots per explant and maximum length of shoots 5.13 ± 0.14 cm resulted (Table 1; Fig. 1B)

Axillary shoot proliferation in some species may be promoted by the presence of an auxin and cytokinin. Assuming that combined effect of auxin and cytokinin could improve further multiplication rate of shoots, different concentrations and combinations were studied. In the present study various concentrations of BAP in combination with different auxins such as NAA, IAA and IBA were used with MS media for optimizing shoot regeneration. Combination of BAP (2.0 mg/l) + NAA (0.5 mg/l) with 5.72 ± 0.04 shoots per explant and maximum length of shoots 4.16 ± 0.16 cm was found to be optimal (83%) for shoot proliferation from nodal explants of *Boucerosia indica* among different combinations of cytokinin and auxins.

Normally for the culture of cells, tissues and organs it is necessary to incorporate a carbon source into the medium. Sugars mainly act as a carbon source but part of its effect can sometimes shown to be due to the provision of a medium with suitable water potential. Among the five carbon sources, sucrose proved to be better for shoot regeneration followed by fructose, glucose, maltose and galactose in this plant. In *Boucerosia indica* explants cultured with 3% sucrose produced maximum number of 4.60 + 0.16 shoots per explant with an average shoot length of 2.82 + 0.06 cm and 80% response.

Callus culture and organogenesis studies

Various types of media such as MS, B5 and WPM were used for callus induction from mature internodal segments of *Boucerosia indica* and improved response was noticed on MS medium than B5 and WPM. Internodal segments of *Boucerosia indica* were cultured on MS medium supplemented with different auxins like 2,4-D, 2,4,5-T and Picloram. Among all the auxins tested, 2,4-D 2.0 mg/l was found to be effectual (87%) for callus induction in *Boucerosia indica* than 2,4,5-T and Picloram (Table 2; Fig. 2 C). Further experiments were carried out with 2.0 mg/l 2.4-D. Inter nodal explants were cultured on MS basal medium supplemented with 2,4-D 2.0 mg/l in combination with BAP, Kn, 2-iP. In *Boucerosia indica* callus proliferated on 2,4-D 2.0 mg/l + BAP 1.0 mg/l (84%) was better than other auxin and cytokinin combinations.

In vitro organogenesis or callus regeneration depends on the application of exogenous phytohormones. Auxins and cytokinins are able to bring shoot or root formation from callus but the effective concentrations of these regulators may vary. Subculture of above calli in case of *Boucerosia indica*, induced with BAP 2.0 mg/l (70%) was best selected for organogenesis or regeneration (Fig. 2 F).

Most workers have found that the concentration of NO^{-3} and NH^{+4} ions in MS medium have been critical for callus initiation and shoot formation. As MS medium responded better, it is selected for further callus studies as well as organogenesis. MS medium was found to be effective for callus induction in other members

of Asclepiadaceae such as *Caralluma pauciflora* (Kiranmai 2006) and *Caralluma* species (Aruna 2006). Based on the percentage of response and the rate of callus induction with auxins was found to be optimum above and below this concentration, the rate of callus induction or percentage of callus responding decreased. The effectiveness of 2,4-D in inducing callus was reported by Sharma and Chandel (1992) and Sharma *et al.* (1995). Occurrence of green spots, which are considered meristematic centres, can be predicted as the capacity of callus to produce shoots. Thus the manipulatable nature of tissue culture can be exploited for the physiological analysis of regeneration or organogenesis *in vitro*. The potential of a medium with only a cytokinin for the induction of callus has been reported in *Tylophora indica* (Manjula *et al*. 2000).

In vitro rooting and acclimatization

After 6 - 8 weeks, when regenerated shoots attained a length of more than 4 cm, they were excised and inoculated on different ½ strength media such as MS, B5 and WPM to evaluate best medium for *in vitro* rooting of micro shoots. Among the various media tested ½ MS medium was proved to be effective for *in vitro* rooting in *Boucerosia indica*. Commonly treatment with PGRs considerably speeds the process of rooting to produce more roots. The trick in obtaining well-rooted plants is to keep callus growth to a minimum. After transferring shoots to root induction medium they should not be deteriorated during incubation. Half strength MS medium was supplemented with three auxins such as IAA, IBA and NAA at different concentrations. Half strength MS medium fortified with NAA 0.1 mg/l with 9.57 ± 0.05 roots per explant and maximum length of roots 3.21 ± 0.12 cm was effective for better rooting (75%) in *Boucerosia indica* (Table 3; Fig. 3 C,D).

Concentration of mineral salts in the medium play a vital role in root induction, high salt levels are frequently inhibitory to root initiation. The favorable effect of low concentration of macro and micronutrients on rooting is probably due to decreased requirement of nitrogen for rhizogenesis in the present study in this plant. Auxins played a crucial role in root induction. Difference was noticed in the nature of roots induced depending on the auxins used in the medium. The reason for the reduced survival in higher concentrations of auxin treatments may be the reason of poor vascular connection of the root with the stem because on the intervention of callus. The positive response of rooting in the present study is similar to other members of Asclepiadaceae such as *Caralluma sarkariae* (Sreelatha *et al.* 2009), three varieties of *Caralluma* adscendens (Aruna *et al.* 2009).

The transplantation stage continues to be a major step in the micropropagation of plants. The advantage of any micropropagation system can however only be fully realized by successful transfer of plantlets from tissue culture vessels to natural conditions or ex vitro. Acclimatization of *in vitro* plantlets to green house or field condition is a critical step for many plant species. So *in vitro* grown plantlets require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil (Fig. 3 G,H). The rooted plants were successfully established and acclimatized in soil with 85% survival rate in *Boucerosia indica.*

Plant tissue and cells in culture illustrate distinction and this appears to be an unpredictably wealthy and noble source of genetic variability. Tissue culture or *in vitro* propagation is one of the fundamental tools for multiplication of endangered medicinal plants. Application of plant tissue and organ culture has immense potential in the large-scale propagation and conservation of the biodiversity of plants. In this study efforts were made to standardize a micropropagation protocol using mature explants of *Boucerosia indica.* It is hoped that such a protocol will provide the planting material to replenish the

declining natural stocks of these plants.

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