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In Vitro Propagation of Croton Scabiosus Bedd. (Euphorbiaceae), an Endemic and Vulnerable Tree Species

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Abstract

Croton scabiosus Bedd. (Euphorbiaceae), an endemic tree species of Southern Eastern Ghats of Andhra Pradesh, India, categorised as 'Vulnerable' is attempted for *in vitro* propagation. Seed pathological problems, poor germination, recurrent fires and tree fellings in the native habitat are the major threats of the species. With an objective to standardise a suitable multiplication protocol to augment the species population in the natural habitat, the present study focus on multiplication of the species through micropropagation. Of the various growth regulators employed in the experimentation, maximum mean number of shoots i.e. 5.37±0.12 was found on MS medium fortified with 0.5mg/l BAP and 2.5mg/l IAA. Maximum mean length of shoots 5.27±0.14 was found on 0.5mg/l BAP+1mg/l IAA. *In vitro* rooting was found on half strength MS medium fortified with 2.5mg/l IBA (8.92±0.19 mean number of roots with 5.04±0.05 mean length of roots with 90% response). Only 10% of survival rate was observed and the species warrants effective on site conservation methods.

Key words

Croton scabiosu; endemic and vulnerable species; conservation; *in vitro* propagation.

Academic Discipline And Sub-Disciplines

Plant Sciences, Conservation.

Subject Classification

Plant Tissue Culture, *In vitro* Propagation.

Type (Method/Approach)

Experimental.

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Introduction

Trees, the dominant life form of the tropical forests, are of exceptional ecological importance as they provide habitat for a wide range of other life forms and their benefits to humanity are multifarious. Small trees especially in arid and semi arid forest habitats have critical role at local and regional level in forest conservation and management; carbon sequestration and consequently climate change. High species diversity of the forest depended on small trees and these plants have been reported to be a major component of forest ecosystems and play an important role in many ecological functions and processes (Yirdaw 2001; Nagaike et al. 2006) Conservation of small trees is indispensable for sound maintenance of forests (Hagihara et al. 2008). It is generally recognized that the most effective way to ensure the long-term survival and evolution of tree species, and the ecological communities of which they are a part, is to maintain viable populations in their native environment (Kramer et al. 2011) and studies in this line holds immense significance.

In recent years, increasing emphasis has been placed on integrated conservation approaches, in which *in situ* and *ex situ* are combined, often together with reintroduction and ecological restoration (Oldfield and Newton 2012). Especially *ex situ* measure is important for conserving endemic and threatened species with low seed germination. *In vitro* propagation can provide plants for reintroduction and research when traditional propagation methods are not adequate (Pence 2013). For many threatened species, traditional propagation by seed or cuttings can meet propagation needs, but for species with few or no seeds or few individuals, plant tissue culture can be used to supplement these methods (Sarasan et al. 2006). Micropropagation has been a great potential tool to overcome problems related with the field culture for plants that do not have regenerative capacity in natural conditions because of various intrinsic and extrinsic factors (Hidaka and Omura, 1989). Micropropagation work is generally carried out for all those trees that have less than 50% seed germination and has poor regeneration potential.

Euphorbiaceae, a dominant tree family of India (Chakrabarthy and Balakrishnan 2012) comprises species that are well known for their medicinal, timber, ornamental and other ecosystem values. Owing to their importance, many workers attempted to propagate different species *in vitro*. This include *Sapium sebiferum* (Varsha 1983), *Euphorbia lathyris* (Ripley and Preece 1986), *Cleistanthus collinus* (Quraishi et al. 1996; Quraishi and Mishra 1998), *Glochidion multiloculaire* (Yamuna et al. 1995), *Excoecaria agallocha* (Rao et al. 1998; Arumugam and Panneerselvam 2012), *Uapaka kirkiana* (Maliro and Kwapata 2000), *Euphorbia tirucalli* (Uchida et al. 2004) *Euphorbia nivulia* (Martin et al. 2005), *Mallotus repandus* (Kaewsuwan et al. 2005; Prathanturarug et al. 2007), *Givotia rottleriformis* (Rambabu et al. 2006) and *Phyllanthus acidus* (Duangporn and Siripong 2009).

Croton is one of the largest genus of family Euphorbiaceae; many authors attempted *in vitro* propagation of in different species of the genus. Micropropagtion of *Croton sublyratus* was made successful through shoot tip culture by Shibata et al. (1996). Lima et al. (2008) induced callus from *Croton urucurana* leaf explants. Pemila and Iriawati (2010) propagated *Croton tiglium* through tissue culture. Ashish and Sharma (2011) worked on *Croton bonplandinus* and succeeded in multiplication of the species. Oliveira de et al. (2011) studied micropropagation of *Croton antisyphiliticus* and Ortiz da Silva et al. (2013) propagated *Croton floribundus* for secondary metabolite production through leaves.

Croton scabiosus Bedd. a small deciduous tree is the candidate species for the present work is strict endemic to deciduous forests of southern Eastern Ghats of Andhra Pradesh (Anantapuram, Kadapa, and Nellore districts). Apart from the ecological values, the species has medicinal value (Venkata Ratnam and Raju, 2005) and different vegetative parts of the species are rich in secondary metabolites (Sarojini Devi et al. 2011). The species populations are threatened in the native habitats primarily by recurrent fires and secondarily by seed pathology, poor germination and tree fellings. Based on field observations, Salamma (2014) categorized the species as vulnerable and opined *in vitro* propagation is one of the options for propagating the species and augmenting the natural population.The main objectives of this study is to evaluate a standard protocol for *in vitro* plantlet production to augument the species population in the natural and allied habitats.

1.Materials and Methods

All the operations of plant tissue culture were carried out in Plant Tissue Culture Laboratory, Department of Botany, Sri Krishnadevaraya University, Anantapuram, Andhra Pradesh - India.

1.1. Materials

Source of explants

Successful *in vitro* studies of any species could be possible through the usage of various explants like leaf, shoot tips, hypocotyls, cotyledons, roots, anthers, seeds, embryos and etc. Juvenile plant parts and fruits of *C. scabiosus* were collected from the representative localities in dry deciduous forests of Anantapuram (Chinna palle, 14°18'37.6"N 78°13'59.6"E at 648m), Kadapa (Sanipayi 14°07'20.4"N 78°54'48.6"E at 614m) and Nellore (Pandrangi 14°55'57.4"N 79°10'29.3"E at 346m) districts of Andhra Pradesh State and experimented to select suitable explant source through study of morphogenetic reponse of nodes, internodes, seeds and embryos. Explant material was carried to laboratory by using wet cotton method. Representative specimens of the species from all the localities were deposited in S.K. University Herbarium (SKU) Anantapuram.

Methodology

Media formulation was carried by using three types of media i.e. MS (Murashige and Skoog 1962), B5 (Gamborg et al. 1968) and WPM (Lloyd and Mc Cown 1981). Media was prepared by amending 3% of Sucrose (Merck, India) along with different concentrations of auxins and cytokinins. The p^H was adjusted to 5.6-5.8 with 1N NaOH, and 1N HCL, then

0.8% of agar-agar (Hi Media, India) was added and melted at 100º C for 2-3 minute. Then the media was suspended into the culture vials and autoclaved at 121º C at 15 lbs pressure for 15 minute for sterilization, then tubes were placed in a cool chamber for solidification.

1.2. Surface sterilization of explant source

Tender leaves, petioles, inter-nodes, shoot tips and nodes were taken for surface sterilization. The plant material was thoroughly washed with running tap water for more than 15 minutes to remove all extraneous particles. For juvenile parts, 2-4 drops of Labolin and for fruits, 2 drops of Tween-20 was used; the leftovers were completely removed by thorough washing. The remaining sterilization procedure was carried out in LAF cabinet. The explants material was washed with Millipore water to remove all the remnants of the surfactants.

Sterilization of plant material was tested with three different sterilants: Mercuric Chloride ($HgCl_2$) at 0.1%, 0.3%, 0.5%, 1.0% concentrations; Sodium Hypochlorite (NaoCl) at 5%, 10%, 20%, w/v and Hydrogen Peroxide (H_2O_2) 10% and 15%, 30%. Since the source material is collected from natural population, 70% alcohol was used for sterilization for about 60 seconds to arrest the contaminants completely. Finally the plant material was rinsed with Millipore water for 4-5 times.

1.3. Usage of plant growth regulators

MS basal medium supplemented with BAP, Kn, IAA, 2- ip, NAA, IBA, DICAMBA, 2, 4-D, 2,4,5-T in 0.5, 1, 1.75, 2.5, 3.5mg/l concentrations were used throughout the study in single and combinational modes. MS medium fortified with 0.5mg/l BAP has shown 80% regeneration from embryos and 70% response from mature nodal explants. Hence, this was maintained as standard concentration to establish hormonal combinations for shoot production.

1.4. Inoculation and incubation of the explants

Surface sterilized explants were transferred to the sterilized Petri plates with filter papers and both ends were excised. Then the explants were trimmed into small pieces in 2cm diameter. The excised explants were cultured on full strength MS medium (Murashige and Skoog, 1962) in culture tubes (Borosil make) and then incubated at 25ºC±1ºC with 16 hrs photoperiod and 8 hrs of darkness at 2000 lux of light intensity.

1.5. Shoot induction, root formation and acclimatization

Mature and *in vitro* grown explants derived from embryo culture were transferred to the medium fortified with single and double combinations of phyto harmones in different concentrations. Further results were registered base on the length, number of shoots per explants and number of explants responded. For *in vitro* rooting, shoots with 2-3 nodes were cultured on different media fortified with 2% sucrose and 0.6% agar. After establishing suitable medium, it was augmented by various concentrations and combinations of auxins like IAA, IBA, NAA and with other cytokinins.

Well rooted *in vitro* grown plantlets were taken out and washed with running tap water, sterilized double distilled water to remove medium residues and transferred to plastic pots. Prior to this, light intensity was adjusted to 12 hours and the temperature was maintained as 27±2ºc. Some of the rooted plantlets were transfer to plastic pots filled with peat moss, garden soil and manure mixed in 1:1:1 ratio and the remaining are transferred to pots containing the soil collected from natural habitats mixed with 1:1 ratio of manure and peat moss. These pots are covered with polythene material and incubated in culture room for more than a week. The pots were irrigated with ½ MS medium devoid of sucrose in alternative days. Small perforation was made on the polythene cover at one edge to make the humidity inside the cover close to outer humidity. Perforation size was made larger to balance the humidity of outer and inner environment.

Statistical analysis of data

One culture tube with single explants was considered as one replicate and each experiment carried 10 replicates and each experiment was repeated for 3-5 times. The data was statistically analyzed through DMR test at the 0.05% level of significance. Standard error of means was calculated for each experiment.

2. RESULTS

2.1 Evaluation of explants

Nodal segments collected from Chinna Palle area has shown only 40% response; Sanipaya area, about 60% and Pandrangi only 20% (Fig.1). Embryos isolated from the seeds of plants of Chinna Palle area responded to about 30%; Sanipaya, about 81% and Pandrangi about 60% (Fig.1). Hence, mature nodal explants and embryos from Sanipayi area are found suitable for further experimentation.

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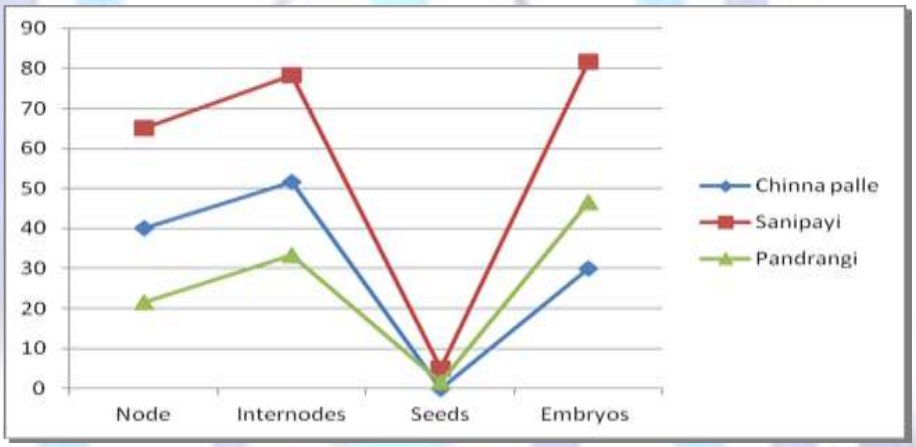


Figure1: Morphogenetic response of explants collected from different localities

2.2. Standardization suitable sterilent

In the present investigation, it is noted that 0.1% $HgCl_2$ is an appropriate sterilant with 1 minute exposure at which healthy shoot were produced (75%) and further growth was not affected by any contaminants

2.3. Shoot initiation

Shoot induction was achieved by two explants i.e. nodes and embryo.

Embryo culture

Results revealed that MS+0.5mg/l BAP+3mg/l IAA showed 90% of embryo development and four specific observations were also recorded: more than 60% of embryos are able to form shoot and root simultaneously; 20.7% of embryos showed root formation; approximately 11.7% embryos formed shoots; of the embryos responded, 6.4% proliferate callus (Salamma and Ravi Prasad Rao, 2013b). The main objective of embryo culture is to produce plantlets for shoot induction *in vitro*.

Effect of cytokinins and its combinations on shoot proliferation

Mature explants responded well on MS+0.5mg/l BAP with 70% shoot sprouting frequency and 4.21±0.10 mean number of shoots and 1.80±0.35 mean length shoots (Table 1; Fig. 2A). Whereas MS+1mg/l BAP has shown high mean of shoot length than any other concentrations (Fig. 2B). MS+1.75mg/l 2 ip has affected the explants to exhibit 2.54±0.32 mean number of shoots and 0.98±0.53mean length of shoots (Fig. 2C). The results pertaining to response of shoot regeneration from hormonal combinations, MS+0.5mg/l BAP+0.5mg/l Kn has shown good result by performing 73.3% of shoot sprouting frequency and 4.52±0.1 mean number of shoots. Mean of length i.e. 2.00±0.35 was observed at the same concentration (Table 1). High mean length of shoots (2.50±0.84) was observed on MS+0.5 BAP+1.0 Kn in mg/l (Fig. 3E). As single supplement Kn has not affected the inoculums but when used in combination with other hormones it was found affective on multiple shoot formation. MS+0.5mg/BAP+2.5mg/l Kn has produced second highest number of shoots. The high mean length of shoots (2.50±0.84) was observed on MS+0.5 BAP+1.0 Kn in mg/l (Table 1).

A B C

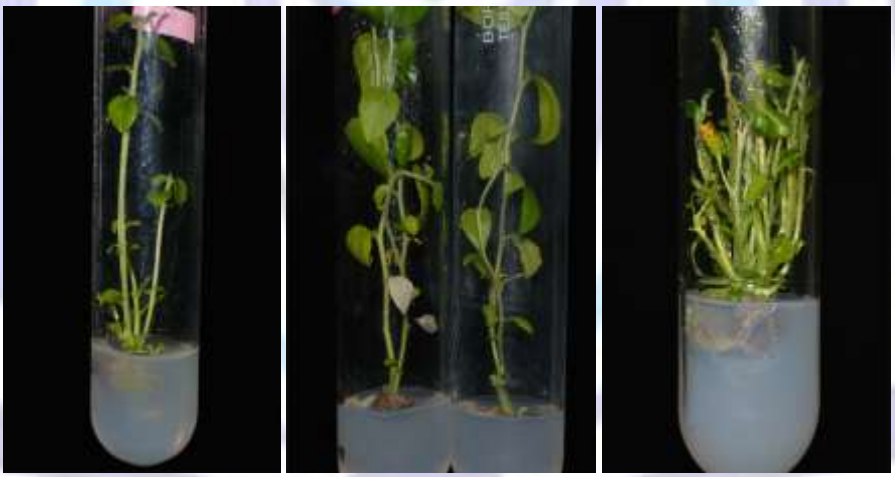
Figure 2: Shoot initioation and multiplictaion

A. Healthy shoots on MS+0.5mg/l BAP
B. Elongated shoots from mature explants on MS+1mg/l BAP
C. Shoot induction from mature explants on MS+1.75mg/l 2 ip

Effect of cytokinin and auxin combination on multiple shoot formation

Evaluation of effect of hormonal combinations was tested with 7 sets of hormonal combinations with BAP, Kn, NAA, IAA, IBA, 2, 4-D (Table 2). The present experiment was initiated by using BAP and IAA based on the former results tabulated in table 2. Maximum mean number of shoots i.e. 5.37±0.12 was found on MS medium fortified with 0.5mg/l BAP and 2.5mg/l IAA with 73.3% of sprouting frequency (Fig.3D). Maximum mean length of shoots per explant (5.27±0.14) was on 0.5mg/l BAP+1 mg/l IAA (Table 2) along with 51.1% of sprouting frequency. MS medium enriched with 0.5mg/l Kn +0.5mg/l 2, 4-D has proliferated least number of shoots i.e. 0.08±0.01, with sprouting frequency of 64.4%.

On the other hand, 0.5mg/l BAP and 2.25mg/l NAA, 0.5mg/l BAP+2mg/l 2.4-D has shown no response (Table 2). But very nearby concentration of 2, 4-D i.e. 1.5mg/l along with 0.5mg/l BAP has delivered good results with 23.3% of sprouting frequency and 3.80±1.21 of mean number shoots per explant and also 2.56±0.02 of mean length of shoots per explant. Regarding NAA, 4.07±0.06 mean number of shoots was formed on MS+0.5mg/l BAP+1mg/l NAA with 33.3% sprouting frequency (Table 2).



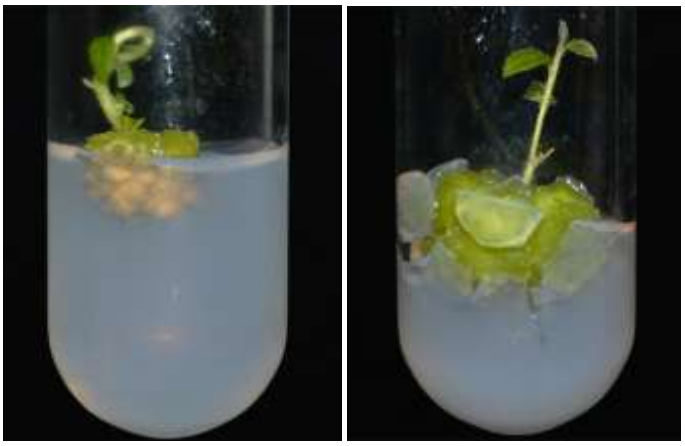
D E F

Figure 3: Multiple shoot formation from hormonal combinations

D. Multiple shoots formed from MS+0.5mg/l BAP+ 2.5mg/l IAA
E. Two replicates showing multiple shoots on MS+0.5 mg/l BAP +1mg/l Kn
F. Multiple shoot prodution on MS+0.5mg/l+2.5 mg/l Kn+ 1.75mg/l 2 ip

2.3. Callus induction and organogenesis

Either shoots or roots can be obtained by organogenesis from callus. The percentage of organogenesis was tested in the present study by using BAP, Kn, 2 ip. Results revealed that 1.75mg/l BAP and 1.75mg/l 2 ip fortified to MS medium has shown good result (2.13±0.12 mean number of shoots (Table 3, Fig.4G) with 72% of response (Table 3). On MS medium fortified with 0.5mg/l Bap+1ml/l Kn +2.5mg/l 2ip has shown 1.64±0.19 mean number of shoots from the calli (Fig.4H). Very poor effect was rendered by 0.5mg/l BAP and 0.5mg/l 2 ip as the response was only 0.07±0.10 mean number of shoots.



G H

Figure 4: Organogenisis from callus on different harmonal combinations

G. Single shoots originating from MS+1.75mg/l BAP+1.75mg/l 2 ip
H. Elongated healthy shoot on MS+0.5mg/l BAP +1mg/l Kn+ 2.5mg/l 2 ip

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Table 1: Effect of various cytokinins, and cytokinin combinations (Bap, Kn, 2 ip) on shoot formation of *In vitro* grown nodal explants of *Croton scabiosus*

Conc. Used (in. mg/l)	No. of inoculums	No. of responded inoculums	SSF (%)	MNS ±SE	MLS± SE	Plant growth regulator			No. of inoculums	SSF (%)	MNS± SE	MLS ± SE
BAP						**BAP**	**Kn**	**2 ip**				
0.5	40	28	70	4.21±0.10	1.80±0.35	0.5	0.5	--	30	73.3	4.52±0.1	2.00±0.35
1	40	18	45	2.03±1.00	2.50±0.84	0.5	1	--	30	53.3	2.03±1.0	2.50±0.84
1.75	40	22	55	1.42±0.32	0.69±0.45	0.5	1.75	--	30	46.6	1.42±0.32	0.69±0.45
2.5	40	15	37.5	3.52±1.56	1.98±0.01	0.5	2.5	--	30	65.4	3.52±1.56	1.98±0.01
3.5	40	11	27.5	1.57±0.41	2.01±0.14	0.5	3.5	--	30	13.3	1.57±0.41	2.01±0.14
Kn												
0.5	40	--	--	NR	NR	--	2.5	0.5	30	53.3	2.34±1.04	1.42±0.02
1	40	12	30	2.41±0.23	1.78±0.36	--	2.5	1	30	36.6	1.79±0.12	0.79±1.2
1.75	40	8	20	1.49±0.12	1.96±0.38	--	2.5	1.75	30	26.6	2.63±0.29	1.23±0.45
2.5	40	--	--	NR	NR	--	2.5	2.5	30	44.4	0.4±0.01	0.66±0.9
3.5	40	15	37.5	0.46±0.01	1.23±0.06	--	2.5	3.5	30	56.6	1.89±0.03	1.52±0.03
2 ip												
0.5	40	9	22.5	0.56±0.42	0.27±0.11	1	--	0.5	30	63.3	0.08±0.0	1.23±0.02
1	40	14	35	1.28±0.32	1.58±0.28	1	--	1	30	53.3	1.41±0.23	0.56±1.02
1.75	40	12	30	2.54±0.32	0.98±0.53	1	--	1.75	30	60	2.31±1.04	1.94±1.04
2.5	40	6	15	1.89±0.02	1.73±0.31	1	--	2.5	30	30	2.56±1.20	2.32±1.02
3.5	40	17	42.5	1.64±0.12	0.86±0.3	1	--	3.5	30	36.6	1.67±0.74	0.78±0.07
--	--	--	--	--	--	0.5	2.5	1.75	30	66.6	3.52±1.06	1.98±0.01
--	--	--	--	--	--	0.5	2.5	1.75	30	26.6	1.94±0.02	2.32±0.12
--	--	--	--	--	--	0.5	2.5	1.75	30	46.6	2.07±0.01	1.47±1.02
--	--	--	--	--	--	0.5	2.5	1.75	30	56.6	0.70±0.01	1.86±1.12
--	--	--	--	--	--	0.5	2.5	1.75	30	40	2.8±1.03	1.2±10.02

MNS- Mean number of shoots, MLS- Mean length of shoots, SE- Standard error, SSF-Shoot Sprouting Frequency

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Table 2: Effect of various combinations cytokinin and auxin on shoot multiplication from *in vitro* grown nodal segments of *Croton scabiosus*

S. No	Conc. Used (mg/l)	SSF (%)	MNS ±SE	MLS±SE
1	**MS+BAP+IAA**			
	0.5+ 0.5	44.4	4.5±1.02	3.01±0.01
	0.5+1	51.1	2.74±0.14	5.27±0.14
	0.5+1.75	66.6	3.05±0.01	4.04±0.10
	0.5+2.5	73.3	5.37±0.12	2.59±0.21
	0.5+3.5	68.8	2.75±0.03	3.79±0.17
2	**MS+IAA+ Kn**			
	0.5+0.5	46.6	1.13±0.01	1.02±0.12
	0.5+1	61.2	3.92±0.10	1.99±0.02
	0.5+1.75	55.5	4.32±0.19	2.87±0.01
	0.5+2.5	82.2	2.01±0.07	3.54±0.11
	0.5+3.5	35.5	2.03±0.01	4.01±0.09
3	**MS+Kn+2,4-D**			
	0.5+0.5	64.4	0.08±0.01	1.23±0.02
	0.5+1	57.7	1.29±0.03	1.58±0.17
	0.5+1.75	33.3	1.03±0.01	3.27±0.14
	0.5+2.5	26.6	3.19±0.01	2.98±0.02
	0.5+3.5	42.2	1.67±0.74	0.78±0.07
4	**MS+ Kn+ IBA**			
	0.5+0.5	73.3	2.01±1.03	3.04±1.25
	0.5+1	51.1	1.41±0.23	0.56±1.02
	0.5+1.75	24.4	2.56±1.20	2.32±1.02
	0.5+2.5	40	3.24±0.09	2.03±0.02
	0.5+3.5	17.7	2.31±1.04	1.94±1.04
5	**MS+BAP+NAA**			
	0.5+0.1	40	1.34±0.56	1.50±0.16
	0.5+0.5	66.6	3.23±0.05	1.22±0.18
	0.5+1	33.3	4.07±0.06	1.24±0.22
	0.5+1.75	26.6	1.50±0.13	3.09±0.06
	0.5+2.25	NR	NR	NR
	0.5+3.5	76.6	2.17±0.34	1.53±0.22
	0.5+4.25	46.6	0.76±0.02	1.01±0.03
6	**MS+BAP+2 4-D**			
	0.5+0.1	27.2	2.97±1.17	1.48±0.11
	0.5+0.5	20	2.01±0.18	3.42±0.04
	0.5+1.5	23.3	3.80±1.21	2.56±0.02
	0.5+2	NR	NR	NR
	0.5+2.75	30	2.39±0.05	1.12±0.01
	0.5+3.25	46.6	3.09±0.06	2.52±0.03
	0.5+4.25	20	0.09±0.01	1.04±0.01
7	**MS+BAP+IBA**			
	0.5+0.1	76.6	2.89±0.72	1.79±0.04
	0.5+0.5	23.3	3.62±0.13	2.23±0.06
	0.5+1	40	4.24±0.01	1.23±0.15
	0.5+1.5	46.6	3.78±0.04	2.46±0.02
	0.5+2.5	26.6	2.97±0.08	1.76±0.17
	0.5+3.25	30	1.90±0.02	1.92±0.01
	0.5+4.25	23.3	0.05±0.01	1.23±0.12

Table 3: Effect of different types of hormonal combinations on morphogenetic response of callus induced from mature internodal explants of *Croton scabiosus*

Type of Cytinkinin/ Conc. (in.mg/l)			No. of responded inoculums	% of response	MNS±SE
BAP	**Kn**	**2 ip**			
0.5	-	0.5	12	48	0.07±0.10
1	-	1	14	56	1.02±0.01
1.75	-	1.75	18	72	2.13±0.12
2.5	-	2.5	9	36	1.76±0.01
3.5	-	3.5	-	-	-
-	0.5	0.5	10	40	1.10±0.09
-	1	1	-	-	-
-	1.75	1.75	14	56	1.01±0.19
-	2.5	2.5	17	68	1.29±0.12
-	3.5	3.5	5	20	2.01±0.13
0.5	1	0.5	4	16	1.78±0.23
0.5	1	1	11	44	1.06±0.04
0.5	1	1.75	13	52	2.06±0.16
0.5	1	2.5	8	32	1.64±0.19
0.5	1	3.5	-	-	-

3. In vitro rhizogenesis

Effect of auxins and its combinations on in vitro rooting

Half strength MS medium is found suitable as the explants have responded well. IBA has shown the best results of rooting. MS+2.5mg/l IBA is able to produce 8.92±0.19 mean number of roots with 5.04±0.05 mean length of roots with 90% response of all the inoculated inoculums (Fig. 5). On ½MS+0.1mg/l IAA the shoots were able to induce 73.3% of rooting with 5.01±0.19 mean length of roots.

Positive results were gained on ½MS+0.5mg/l IAA+2.5mg/l IBA i.e. 7.34±0.06 mean number of roots with 5.51±0.02 mean length of the roots (Fig. 6J). Other hormones like 2, 4-D, IBA, IAA and NAA has shown considerable results, 1.75mg/l 2, 4-D+1.75mg/l IBA has proliferated 5.62±0.07 mean number of roots with 4.09±0.06 mean length of roots (Table 4, Fig. 6K). Poor results of 1.24±0.05 mean number of shoot with 2.16±0.12 were obtained on ½ MS medium supplemented with 1mg/l NAA and 1mg/l IAA. Half strength MS fortified with 0.5mg/l IAA+1mg/l IBA, ½MS+0.5mg/l 2,4-D+0.5mg/l IBA and 3.5 mg/l NAA+ 3.5 mg/l IAA were not rendered any influence on shoots to produce roots (Table 4).

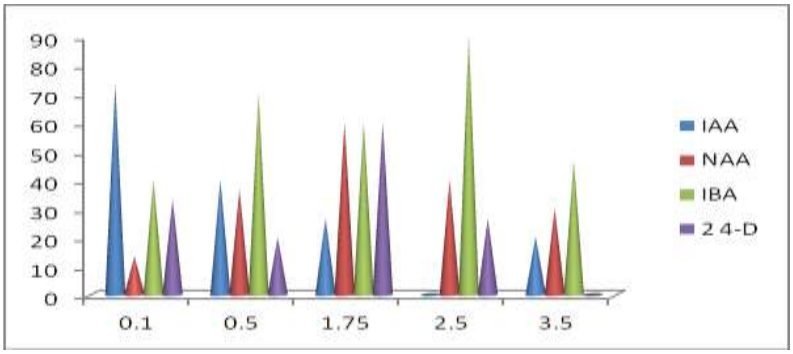

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Figure 5: Effect of different concetrations of auxins on rooting efficiency of in vitro grown shoots of *Croton scabiosus*

I J K L

Figure 6: Root induction as well multi root formation on ½ MS medium with different harmonal cambinations

I. Only root formation on ½MS+0.5mg/l IAA+3.5 mg/l IBA
J. Profuse root formation ½MS+0.5mg/l IAA+2.5mg/l IBA
K. Rooting on ½MS+1.75 mg/l 2,4-D+1.75mg/l IBA
L. Complete and healthy plantlet on ½ MS+0.5mg/l NAA+0.5mg/l

4. Acclimatization of plantlets

The well rooted plantlets were transferred to the plastic pots. Incubated plantlets can be seen in Fig. 7M. Plantlets transferred to the pots having 1:1:1 ratio of garden soil, peat moss and manure were not able to survive but plantlets transferred to the soil collected from the natural habitats were survived. However, these pots when transferred to the room conditions with cover, survived for 3-4 days and removal of the cover made the plantlets to feel sensitive to the outer environment. At the end only 10 out of 50 were survived. Of the 10 plantlets transferred to green house conditions most of them were wilted and only one plantlet is survived. Thus the survival rate at the end is only 10% (Fig. 7 N, O).

M

N O

Fig. 7: Acclimatization and establishment of plantlets

M. Pots under acclimatization process in Culture room conditions

N. Acclimatized plant in green house conditions

O. Single acclimatized plant of *Croton scabiosus* in SKU botanic garden

Table 4: Effect of various auxin combinations on rhizogenesis from in *vitro* rose shoots of *Croton scabiosus* on half strength ms medium

Combination of hormones	Conc. (in. mg/l)	Response (in. %)	MNR± SE	MLR±SE
IAA+IBA	0.5+ 0.5	60	5.25± 0.14	0.5±0.01
	0.5+1	NR	NR	NR
	0.5+1.75	56.6	3.52±0.02	2.00±0.04
	0.5+2.5	36.6	7.34±0.06	5.51±0.02
	0.5+3.5	23.3	2.19±0.09	3.01±0.12
NAA+ IBA	0.5+0.5	73.3	5.38±0.12	1.75±0.17
	1+1	62	3.24±0.04	0.9±0.01
	1.75+1.75	86.3	3.54±0.07	1.23±0.14
	2.5+2.5	63.3	5.61±0.03	2.52±0.18
	3.5+3.5	28	1.26±0.01	1.93±0.14
2,4-D+IBA	0.5+0.5	NR	NR	NR
	1+1	40	2.01±0.12	3.09±0.13
	1.75+1.75	33.3	5.62±0.07	4.09±0.06
	2.5+2.5	50	3.54±0.07	1.23±0.14
	3.5+3.5	36.6	2.08±0.12	3.87±0.18
NAA+IAA	0.5+0.5	70	4.19±0.01	3.09±0.08
	1+1	56.6	1.24±0.05	2.16±0.12
	1.75+1.75	76.6	3.25±0.09	1.46±0.14
	2.5+2.5	26.6	2.31±0.14	1.22±0.18
	3.5+3.5	23.3	NR	NR

MNS- Mean number of shoots, MLS- Mean length of shoots, SE- Standard error

Discussion

The explant source for *in vitro* propagation of *Croton scabiosus* is from Sanipaya area, Kadapa district, Andhra Pradesh, Inidia. Mercuric Chloride at 0.1% concentration is found as an appropriate sterilant. The same concentration of $HgCl_2$ with different time exposure was found effective in callus induction of *C. scabiosus* by Salamma and Ravi Prasad Rao (2013a) and in *Jatropha curcus* by Shrivastava and Banerjee (2008). In *Phyllanthus indofischeri* Anitha (2014) used 0.1% of $HgCl_2$ for 5-10 minutes exposure; for regeneration of *Phyllanthus amarus* Chitra et al. (2009) established 0.1% Mercuric Chloride for 2 min for sterilization.

Shoot induction was achieved on 0.5mg/l BAP with maximum response (80%). The same concentration of BAP (0.5mg/l) was also used to propagate *Croton bonplandinus* with nodal explants by Ashish and Sharma (2011). In *Croton scabiosus*, mature explants were responded well on BAP with good shoot sprouting frequency (70%). BAP showed greater number of shoot production from *Baliospermum montanum* (Johnson and Manickam 2003). BAP was utilized in 0.5mg/l BAP for *Phyllanthus amarus* by Mahipal and Dixit (2007).

Regarding the harmonal combinations, BAP with different phyto hormones has shown good impact on different species of Euphorbiaceae. Effective shoot production of *Glochidion multiloculaire* was established by Yamuna et al. (1995) by using MS+1mg/l BA+1mg/l IAA. Shoot proliferation was enhanced with BA at 1µm in *Cleistanthus collinus* (Quraishi et al. 1996); 3.9µ BAP showed maximum shoot induction in *Excoecaria agallocha* (Arumugam and Panneerselvam, 2012.) BA in single and combination with low concentration of auxins produced more number of shoots in *Jatropha curcus* (Shrivastava and Banerjee 2008). In *Baliospermum montanum* MS+0.1mg/l BAP+0.1mg/l NAA showed superior results (Gorge et al. 2008). Our study also achieved good results with BAP in combination with IAA and Kinetin.

Internodal explants on 0.5mg/l DICAMBA has proliferated 87.5% of fresh and green friable callus (Salamma and Ravi Prasad Rao 2013a). Similarly, callus cultures of *Phyllanthus acidus* was established on MS+2, 4-D 1mg/l+ Kn 1mg/l by Duangporn and Siripong (2009). Healthy and high quantities of callus were found in *Croton urucurana* on WPM+2, 4-D (Lima et al. 2008). According to Goerge and Sherrington (1984), combination of auxins and cytokinin promote cellular differentiation and also organogenesis. In our studies, callus was sentenced to organogenesis through BAP with 2 ip (2.13±0.12 MNS), BAP with Kn and 2 ip (2.06±0.16 MNS). Indirect organogenesis has implied from callus cultures of *Phyllanthus amarus* on MS+2mg/l BAP+0.5mg/l GA3 (Chitra et al. 2009).

Rooting was successfully established with different concentrations of IBA on half strength MS medium. In many Euphorbiaceae members, half strength MS medium was found effective in rooting viz. *Cleistanthus collinus* (Quraishi et al. 1996), *Croton tiglium* (Pemila and Iriawati 2010) and *Euphorbia cotanifolia* (Parveen et al. 2013). In many species of Euphorbiaceae IBA has deliberated good results in rooting. Maximum roots were found in ½ MS medium supplemented with 9.8µM IBA in *Baliospermum montanum* (Johnson and Manickam 2003). *In vitro* rooting of *Euphorbia cotinifolia* was successful on ½ MS medium+2.5µM of IBA (Perveen et al. 2013). Effective rooting was established in *Jatropha curcus* on ½MS fortified with IBA in 3.0mg/l (Shrivastava and Banerjee 2008). As stated above, IBA has also shown considerable results in rooting of *Croton scabiosus* both in single (8.92±0.19 MNR, 5.04±0.05 MLR with 90% response) and in combination with other auxins and cytokinins (0.5mg/l IAA+2.5mg/l IBA proliferated 7.34±0.06 MNR and 5.51±0.02 MLR) on half strength MS medium. But in *Croton sublyratus* IBA did not cause substantial enhancement in rooting frequency (Shibata et al. 1996).

Although *Croton scabiosus* responded well in *in vitro* conditions, 10% survival rate of the plantlets developed through micropropagation highlight and warrants the need of *in situ* conservation approaches.

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