Journal of Research in Biology

An International Scientific Research Journal

ISSN No: Print: 2231 –6280; Online: 2231- 6299

Original Research

In vitro multiplication of the endangered plant species, *Exacum bicolor* Roxb

Authors:
Jeeshna MV

Institution:
Department of Botany, Sree Narayana College, Thottada (PO), Kannur, Kerala, India-670 007.

Corresponding author:
Jeeshna MV

ABSTRACT:

To conserve endangered medicinal plant species, *Exacum bicolor* Roxb. (Gentianaceae), an efficient micropropagation protocol was developed by using nodal segments for axillary shoot proliferation. Higher multiplication rate was achieved with 78% on MS medium supplemented with growth hormones, IBA and GA_3 at 1.5 mg/l and 0.5 mg/l respectively. Rooting was more effective with 75% of the node derived *in vitro* shoots in MS basal medium with the auxins, IBA and NAA at 1.0 mg/l and 0.5 mg/l respectively. Using the hardening medium containing garden soil, sand and vermiculate in the ratio of 1:1:1 by volume, regenerated plantlets could be successfully acclimatized at a rate of 80 % survivability.

Keywords:

Micropropagation, *Exacum bicolor,* Shoot proliferation, *in vitro* shoots.

Abbreviations

$HgCl_2$	Mercuric chloride
BAP	6-Benzylamino Purine
2,4-D	2,4-Dichlorophenoxyacetic acid
Kn	Kinetin
GA_3	Gibberellic Acid
NAA	1-Naphthalene Acetic acid
IBA	3-Indole Butyric Acid
IAA	Indole Acetic Acid

Email Id:
mvjeeshna@gmail.com

Article Citation:
Jeeshna MV
In vitro multiplication of the endangered plant species, *Exacum bicolor* Roxb
Journal of Research in Biology (2016) 6(5): 2054-2061

Dates:
Received: 28 Feb 2016 **Accepted:** 12 April 2016 **Published:** 04 July 2016

Web Address:
http://jresearchbiology.com/documents/RA0581.pdf

Journal of Research in Biology

INTRODUCTION

Exacum bicolor (Family: Gentianaceae; Order Gentianales), a well known endangered medicinal herb in the grasslands of Kerala state, India has been used to treat a variety of diseases. In the recent times, phytochemicals from this species are of growing interest in pharmacology due to the usage as a stimulant to diabetics, stomachic and antifungal agent in traditional medicinal practices (Reddi *et al.,* 2005; Pullaiah, 2006; Khare, 2007; Shiddamallayya *et al.,* 2010). Because of this demand, over exploitation resulted in less population size of this species in Kerala (Jeeshna, 2011). In addition to over exploitation by man, very low seed germination rate (<5 %) kept the population size of *E. bicolor* at low level in its grassland habitats at the northern area of Kerala (Jeeshna, 2011). Propagation by stem cuttings is also not effective (Sreelatha *et al.,* 2007). For these reasons, it is difficult to recover the endangered population of this species through conventional propagation methods. Therefore, in the present study, to safeguard this species by increasing its population, an attempt has been made via *in vitro* regeneration by employing tissue culture technology.

MATERIALS AND METHODS

Node from the young and healthy branches of the individuals of the species of *E. bicolor* present in the grasslands of Wayanad district of Kerala, India were used as an explant. The immature nodes had been washed in running water twice and then treated with 5% tween-20 solution for five minutes for surface sterilization and again rinsed in water (Valappil and Subramaniam, 2011). To eliminate the fungal contamination, explants were further treated with 5% antibiotics (ampicillin and rifampicin) for 30 min followed by three rinses in sterile double distilled water.

Figure 1. Micropropagation of *Exacum bicolor*

Table 1. Effect of different concentrations and combinations of growth regulators in the MS medium on callus formation and shoot proliferation from nodal explants of the species, *Exacum bicolor*

Growth regulator (mg/l)				Days required for shoot proliferation after inoculation	Shoot proliferation (%)
BAP	NAA	2, 4-D	Kn	Node	Node
0.5	0.1	0.0	0.0	24	74.35 ^{n} ±0.13
1.0	0.2	0.0	0.0	23	80.37 ^{q} ±0.13
1.5	0.3	0.0	0.0	26	80.07 ^{q} ±0.95
2.0	0.4	0.0	0.0	28	79.41 ^{p} ±0.17
2.5	0.5	0.0	0.0	27	77.29 ^{o} ±0.07
3.0	0.6	0.0	0.0	24	68.46 ^{l} ±0.20
0.5	0.0	0.3	0.0	21	61.58 ^{i} ±0.19
0.1	0.0	0.6	0.0	29	65.52 ^{j} ±0.25
1.5	0.0	0.9	0.0	24	72.59 ^{m} ±0.25
2.0	0.0	1.2	0.0	21	66.64 ^{k} ±0.23
2.5	0.0	1.5	0.0	22	58.50 ^{h} ±0.34
3.0	0.0	1.8	0.0	23	52.38 ^{q} ±0.03
0.0	0.0	0.0	0.3	28	31.34 ^{e} ±0.24
0.0	0.0	0.0	0.6	24	24.44 ^{b} ±0.31
0.0	0.0	0.0	0.9	21	36.64 ^{f} ±0.30
0.0	0.0	0.0	1.2	27	27.37 ^{d} ±0.20
0.0	0.0	0.0	1.5	25	26.60 ^{c} ±0.21
0.0	0.0	0.0	1.8	27	23.40 ^{a} ±0.06

Means followed by different letter in columns are significant to each other according to DMRT at 5% level.

Furthermore, surface sterilization was carried out by dipping the explants in 0.1% $HgCl_2$ for 3 min followed by 3 – 4 rinses in sterilized double distilled water. MS basal medium containing 3% sucrose solidified with 1% agar (tissue culture grade, Himedia, India) had been used. The pH of the medium was adjusted to 5.6 – 5.8 prior to the addition of agar (Padmavathy *et al.,* 2013). The explants were transferred to culture bottles containing 25ml MS basal medium supplemented with different concentrations of the growth regulators BAP and NAA, BAP and 2,4-D and Kn alone for shoot proliferation. Cultures were incubated at 25±2° C under 16 h photo periods from cool white fluorescent tubes giving 2000 lux at the culture level. The shoots that proliferated from primary explants were isolated and subcultured onto the MS medium supplemented with different concentrations of BAP and NAA, BAP and Kn and BAP and GA_3 for bulking up shoot culture material. The green adventitious shoots were subcultured from proliferating cultures and implanted onto the MS medium supplemented with various concentrations of IBA and NAA and IBA and IAA for root induction. The rooted plantlets were then transferred to the hardening media containing different hardening mixtures (Mallikadevi *et al.,* 2008). Triplicates were maintained for all experiments. Data obtained were processed statistically to New Duncan's Multiple Range Test (OMRT) (Gomez and Gomez, 1976).

RESULTS AND DISCUSSION

The effects of cytokinins and auxins at various concentrations on the axillary shoot induction from nodal explants are presented in Table 1. The higher amount of 81.71 % of nodal segments responded well for shoot proliferation within 26 days in the MS basal medium supplemented with BAP and NAA at 2.0 and 0.4 mg/l respectively. The direct shooting of nodal explants were noted in an effective manner while culturing onto the basal medium with the cytokinin, BAP alone. Sarwar *et al.* (2009) already proved the importance of increased cytokinin in shoot development. It has been already reported about the requirement of BAP for effective shoot formation in the members of Gentianaceae, *Swertia chirayita* (Joshi and Dhawan, 2007) and *Exacum*

Figure 2. Serial cultures of *Exacum bicolor in* tissues culture chamber and thawing

travancoricum (Kannan *et al.,* 2007). For instance, BAP was found to be the most effective growth hormone for shoot bud induction in *Chlorophytum borivilianum* (Purohit *et al.,* 1994). Similarly, in *Vitex negundo*, Sahoo and Chand (1998) reported BAP as the most effective growth hormone for shoot bud induction.

The subculturing of node derived *in vitro* produced shoots for multiple shoot induction in the MS basal medium was varied according to the combinations and concentrations of the growth regulators used (Table 2). All subculturing experiments showed that basal medium containing the growth regulators, BAP and GA_3 at 1.5 and 0.5 mg/l respectively exhibited high degree of shooting characters like per cent explant response to shoot initiation (78.14%), number of shoots/ explant (11.17 shoots/explant) and shoot length (6.32 cm). It indicated the fact that high concentration of the growth regulator, cytokinin (BAP) and low concentration of GA_3 were needed for the shoot formation of node derived shoots of this species. Senthilkumar *et al.* (2009) emphasized the importance of BAP and GA_3 for multiple shoot formation in the medicinal plant species, *Ophiorrhiza mungos* of Nilgiris. Several works on the importance of BAP and GA_3 for the multiple shoot formation in different species were already documented well (Ornstrup *et al.,* 1993; Nasirujjaman *et al.,* 2005;

Table 2. Effect of different concentrations and combinations of growth regulators in the MS medium on multiple shooting after sub culturing the node derived *in vitro* produced shoots of the species, *Exacum bicolor*.

Growth regulator (mg/l)				Culture response (%)	Number of shoots/ explant	Shoot length (cm)
BAP	NAA	Kn	GA_3			
0.5	0.3	0.0	0.0	14.19 [a] ±0.13	2.32 [a] ±0.07	2.23 [b] ±0.15
1.0	0.3	0.0	0.0	16.19 [b] ±0.13	3.24 [b] ±0.13	3.13 [c] ±0.08
1.5	0.5	0.0	0.0	22.09 [c] ±0.16	4.25 [c] ±0.09	3.29 [c] ±0.09
2.0	0.5	0.0	0.0	26.17 [d] ±0.21	3.26 [b] ±0.21	1.10 [a] ±0.10
2.5	0.7	0.0	0.0	32.09 [e] ±0.18	5.24 [d] ±0.07	4.20 [d] ±0.11
3.0	0.7	0.0	0.0	48.19 [f] ±0.13	7.25 [f] ±0.09	5.24 [f] ±0.31
0.5	0.0	0.3	0.0	64.12 [j] ±0.14	3.21 [b] ±0.05	2.33 [b] ±0.08
1.0	0.0	0.5	0.0	58.30 [l] ±0.11	4.28 [c] ±0.12	4.19 [d] ±0.14
1.5	0.0	0.7	0.0	49.18 [g] ±0.05	5.33 [d] ±0.07	2.42 [b] ±0.17
2.0	0.0	0.9	0.0	55.81 [h] ±0.38	6.38 [e] ±0.09	3.27 [c] ±0.11
2.5	0.0	1.1	0.0	65.19 [k] ±0.14	8.22 [g] ±0.06	5.24 [e] ±0.08
3.0	0.0	1.3	0.0	68.13 [l] ±0.10	6.27 [e] ±0.11	5.22 [e] ±0.11
0.5	0.0	0.0	0.5	69.23 [m] ±0.09	5.19 [d] ±0.07	4.23 [d] ±0.07
1.0	0.0	0.0	0.5	72.20 [n] ±0.08	10.19 [i] ±0.12	5.24 [e] ±0.08
1.5	0.0	0.0	0.5	78.14 [q] ±0.56	11.17 [j] ±0.10	6.32 [g] ±0.14
2.0	0.0	0.0	0.5	76.20 [p] ±0.04	9.21 [h] ±0.04	5.36 [ef] ±0.07
2.5	0.0	0.0	0.5	74.17 [o] ±0.19	7.23 [f] ±0.09	4.41 [d] ±0.16
3.0	0.0	0.0	0.5	69.24 [m] ±0.08	6.24 [e] ±0.07	4.19 [d] ±0.14

Means followed by different letter in columns are significant to each other according to DMRT at 5% level.

Table 3. Effect of different concentrations and combinations of growth regulators in the MS medium on certain rooting attributes after subculturing the node derived *in vitro* shoots of the species, *Exacum bicolor*

Growth regulator (mg/l)			Shoots rooted (%)	Number of roots/shoot	Root length (cm)
IBA	IAA	NAA			
0.1	0.0	0.0	58.13 [i] ±0.10	5.19 [d] ±0.12	3.22 [b] ±0.05
0.2	0.0	0.0	60.08 [k] ±0.16	6.19 [e] ±0.09	2.29 [a] ±0.11
0.3	0.0	0.0	61.28 [l] ±0.04	4.16 [c] ±0.13	3.18 [b] ±0.05
0.4	0.0	0.0	59 27 [j] ±0.12	3.27 [b] ±0.11	4.19 [c] ±0.04
0.5	0.0	0.0	54.23 [h] ±0.07	3.27 [b] ±0.11	4.24 [c] ±0.07
0.6	0.0	0.0	48.23 [d] ±0.07	2.28 [a] ±0.12	5.18 [d] ±0.17
0.2	0.0	0.1	51.30 [f] ±0.09	7.20 [f] ±0.09	4.19 [c] ±0.03
0.4	0.0	0.2	62.35 [m] ±0.04	5.23 [d] ±0.08	3.10 [b] ±0.08
0.6	0.0	0.3	68.27 [n] ±0.03	7.23 [f] ±0.08	4.30 [c] ±0.08
0.8	0.0	0.4	73.22 [p] ±0.05	9.22 [h] ±0.05	5.53 [e] ±0.25
1.0	0.0	0.5	75.22 [q] ±0.08	10.20 [i] ±0.12	6.00 [f] ±0.08
1.2	0.0	0.6	70.10 [o] ±0.05	8.15 [g] ±0.12	5.00 [d] ±0.08
0.1	0.3	0.0	30.16 [a] ±0.12	6.19 [e] ±0.13	4.51 [c] ±0.42
0.2	0.5	0.0	34.14 [b] ±0.09	4.23 [c] ±0.08	4.24 [c] ±0.09
0.3	0.7	0.0	46.23 [c] ±0.07	5.21 [d] ±0.08	3.18 [b] ±0.05
0.4	0.9	0.0	52.26 [g] ±0.11	6.24 [e] ±0.07	3.31 [b] ±0.25
0.5	1.1	0.0	50.23 [e] ±0.07	4.23 [c] ±0.06	4.23 [c] ±0.08
0.6	1.3	0.0	48.24 [d] ±0.07	3.10 [b] ±0.06	3.16 [b] ±0.10

Means followed by different letter in columns are significant to each other according to DMRT at 5% level.

Joshi and Dhawan, 2007; Vinterhalter *et al.,* 2008; Kurt and Erdag, 2009).

Root formation was induced in *in vitro* regenerated shoots by culturing them on to the MS basal medium containing various concentrations of growth regulators, NAA, IBA and IAA (Table 3). The concentration of IBA and NAA at 1.0 and 0.5 mg/l respectively in the basal medium produced significantly

higher percentage (75.22 %) of roots. It showed that as in many other species, the growth regulator, auxin is playing the most important role for rooting in, *E. bicolor*. Pande *et al.* (2000) and Karuppusamy and Pullaiah (2007), already reported the importance of auxins in the root formation during the subculturing of secondary explants. Similar kind of observations on the requirement of auxins for better rooting were reported in many species (Dewan *et al.,* 1992; Beck *et al.,* 2000; Beck and Dunlop, 2001; Vengadesan *et al.,* 2002, 2003; Nanda *et al.,* 2004; Rout *et al.,* 2008). The rooted plantlets were removed from the basal medium after 20 days of culture and treated with fungicide bavistin (0.4%) and washed thoroughly in sterile double distilled water (http:// shodhganga.inflibnet.ac.in/bitstream/10603/33765/6/ chapter5.pdf). Then they were acclimatized well in the plastic cups containing hardening medium encomposed by garden soil, sand and vermiculate in the ratio of 1:1:1 by volume. About 80% of the plantlets survived and subsequently it has been observed that the plantlets grew well in the greenhouse without any change in phenotypic characters and the clones developed are most identical.

CONCLUSION

The protocol standardized through this study demonstrates a rapid and effective *in vitro* shoot multiplication of *Exacum bicolor* from suitable microsites in the grasslands of northern Kerala.

REFERENCES

Beck SL, Bunlop R and Staden JV. (2000). Meristem culture of *Acacia mearnsii. Plant Growth Regulation*, 32 (1): 49-58.

Beck SL and Dunlop RW. (2001). Micropropagation of the *Acacia* species- A review. *In vitro Cellular and Developmental Biology-Plant*, 37(5): 531-538.

Dewan A, Nanda K and Gupta SC. (1992). In vitro micropropagation of *Acacia nilotica* sub sp. *Indica Brenan. Via cotyledonary nodes. Plant cell Reports*, 12 (1): 18-21.

Gomez KA and Gomez KA. (1976). Statistical procedure for agricultural research with emphasis of Rice. Los Bans. Philipines International Rice Research Institute Book. Los Banos, Philipines. A Wiley-intersclence Publication.

Jeeshna MV. (2011). Evaluation of ecological variants of the endangered plant species, *Exacum bicolor* Roxb. (Gentianaceae) for effective medicinal usage and in vitro regeneration protocol for its conservation. (Dissertation). Bharathiyar University. Coimbatore, Tmil Nadu, India.

Jeeshna Meethaley Valappil and Paulsamy Subramaniam. (2011). Evaluation of certain flavonoids of medicinal importance in the wild and micropropagated plants of the endangered medicinal species, *Exacum bicolor* Roxb. *Journal of Applied Pharmaceutical Science,* 1(5): 99-102

Joshi P and Dhawan V. (2007). Assessment of genetic fidelity of micropropagated Swertia chirayita plantlets by ISSR maker assay boil plant. *Biologia Plantarum,* 51(1): 22-26.

Kannan P, Premkumar A and Ignacimuthu S. (2007). Thidiazuron induced shoot regeneration in the endangered species, *Exacum travancoricum* Beedi. *Indan Journal of Biotechnolgy*, 6(4): 564 – 566.

Karuppusamy S and Pullaiah T. (2007). *In vitro* shoot multiplication of *Bupleurum distichophyllum* Wight – A native medicinal plant of Southern India. *Plant Tissue Culture and Biotechnology,* 17(2): 115-124.

Khare CP. (2007). Indian Medicinal Plants an illustrated dictionary. Springer Science, Business Media, LLC, New York.

Kurt S and Erdag B. (2009). *In vitro* germination and axillary shoot propagation of Centaurea zeybek. *Biologia,* 64(1): 97-101.

Mallikadevi T, Senthilkumar P and Paulsamy S. (2008). *In vitro* regeneration of the medicinal plant, *Plumbago zeylanica* L. with reference to a unique population in Maruthamalai, the Western Ghats, India. *Plant Tissue Culture and Biotechnology*, 18(2): 173-179.

Nanda R, Das P and Rout G. (2004). *In vitro* clonal propagation of Acacia mangium Willd. and its evaluation of genetic stability through RAPD marker. *Annuals of Forest Science*, 61(4): 381-386.

Nasirujjaman K, Salah Uddin M, Zaman S and Reza, MA. (2005). Micropopagation of turmeric (*Curcuma longa* Linn.) through *in vitro* rhizome bud culture. *Journal of Biological Sciences*, 5(4): 490-492.

Ornstrup H, Molgaard JP and Farestveit B. (1993). Somatic embryo-genesis and plant regeneration from cell suspensions of *Exacum affine. Plant Cell Tissue and Organ Culture,* 35(1): 37-40.

Padmavathy K, Paulsamy S and Thambiraj J. (2013). *In vitro* shoot regeneration of *Corchorus acutangulus* Lam. (Tiliaceae). *Research and Reviews: Journal of Botanical Sciences*, 2(3): 1-5.

Pande D, Srivastava PS and Ramasamy NS. (2000). Xanthotoxin in tissue cultures of *Ammi majus* Linn. *Journal of Tropical Medicinal Plants*, 1(1-2): 43-52.

Pullaiah T. (2006). Encyclopaedia of World medicinal plant. Volume 2, Regency pub., West Patel Nagar, New Delhi.

Purohit SDA, Dave A and Kukda G. (1994). *In vitro* conservation strategies for a rare medicinal herb–safed muslii (*Chlorophytum borivilianum* Sant. Etfernand). *Indian Journal of Plant Genetic Resources,* 7(2): 201 – 204.

Reddi STV, Naidu BVAR and Prasanthi S. (2005). In : Herbal remedies for diseases. I Alikhan and AKhanum (Eds), Ukay Publications, Hyderabad.

Rout GR, Senapati SK and Aparajetha S. (2008). Micropropagation of *Acacia chundra* (Roxb.) DC. *Hort Science*. 35(1): 22-26.

Sahoo Y and Chand PK. (1998). Micropropagation of *Vitex negundo* L, a woody aromatic medicinal shrub, through high – frequency axillary shoot proliferation. *Plant cell Reports,* 18(3): 301 – 307.

Sarwar S, Zia M, Rehman R, Fatima Z, Sial RA and Chaudhary MF. (2009). *In vitro* direct regeneration in mint from different explants on half strength MS medium. *African Journal of Biotechnology* 8(18): 4667-4671.

Senthilkumar P, Paulsamy S and Mallikadevi T. (2009). In vitro regeneration of an important medicinal plant, *Ophiorrhiza mungos* L. *Journal of Agriculture, Biotechnology and Ecology*, 2(3): 322-330.

Shiddamallayya N, Yesmeen A and Gopakumar K. (2010). Medico – botanical survey of Kumar Parvatha Kukke Subramanya, Mangalore. *Indian Journal of Traditional Knowledge,* 9(1): 96 – 99.

Sreelatha U, Baburaj TS and Narayan Kutty C. (2007). *Exacum bicolor*- A elegant wild flowering herbs. Underutilized and Underexploited horticultural crops. New India Publishing Agency, New Delhi.

Vengadesan G, Ganapathi A and Anand RP Anbazhagan VR. (2002). *In vitro* propagation of *Acacia sinuata* (Lour.) Merr. *Via cotyledonary nodes. Agroforestry Systems,* 55(1): 9-15.

Vengadesan G, Ganapathi A, Anand RP and Selvaraj N. (2003). *In vitro* propagation of *Acacia sinuata* (Lour.) Merr. from nodal segments of 10 - years old-

tree. *In Vitro Cellular and Developmental Biology - Plant,* 39(4): 409-414.

Vinterhalter B, Jankovic T, Savikin K, Nikolic R and Vinterhalter D. (2008). Propagation and xanthone content of *Gentianella austriaca* shoot cultures. *Plant Cell Tissue and Organ Culture*, 94: 329-335.