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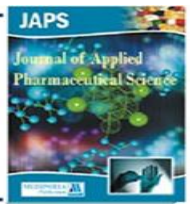

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In Vitro Regeneration of *Exacum wightianum* Arn. (Gentianaceae)- An Endemic Medicinal Plant From The Nilgiris, Western Ghats

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ABSTRACT

Exacum wightianum Arn. (Gentianaceae) is an endemic medicinal plant from the Nilgiri hills, Western Ghats, Tamil Nadu. Indirect regeneration of *E. wightianum* was obtained through organogenesis in callus culture. Axial bud explants were found to be best suited for callus induction on MS medium supplemented with BA + NAA (2.0+0.03 mg/L). Multiple shoots originated from callus obtained from the axial buds. were multiplied by subculture on the same medium. Maximum shoot regeneration was obtained on MS medium supplemented with BA with NAA (2.0+0.5 mg/L), and up to 25shoots was observed within 2 weeks.

Keywords: *Exacum wightianum*, Callus culture, multiple shoot regeneration, BA+ NAA.

INTRODUCTION

Exacum wightianum Arn. (Gentianaceae) is an endemic medicinal *subshrub* plant with erect, branched, compact and stout 4-winged stem distributed in the Nilgiri hills, Southern Western Ghats (Mathew, 1999) about Naduvattam, the Nilgiri Hills, Tamil Nadu, India. The species of the family Gentianaceae are best known for their bitter taste, which can be related to their content of iridoids, such as amarogentin, the bitterest compound known. Bitters have been traditional remedies for loss of appetite and fever, and are still included in many “tonic” formulations (Martindale, 1982). The members of the family Gentianaceae are rich sources of xanthonoids, flavonoids, irridoids and terpenoids. The herbs of this family are extensively used as bitter tonic and febrifuge in the Ayurvedic system of medicine (Nadkarni, 1982). The extracts of a number of species have long been used in folk medicine for the treatment of hepatitis, cholecystitis, pneumonia, malaria, dysentery and spasm; whereas the recent investigations have shown that some xanthones possess a marked hypoglycemic activity when administered to rats. Moreover, some species of *Swertia* are reported to possess CNS-depressant and anti-hepatic principles (Ahmad *et al*., 2002). Xanthone derivatives mangostine, iso mangostine and mangostine triacetate of this family are known to possess significant anti -inflammatory activities, potent anti-platelet activity, anti-cancer and anti- fungal effect (Lin *et al*., 1996). Since this family possesses several medicinal properties, high demand, over exploitations of tribal peoples and poor germination rate, the present study is focused on *in vitro* multiplication of *Exacum wightianum.*

Table 1. Effect of different concentration of growth regulators on callus induction from leaf, node and shoot tip explant of *Exacum wightianum*

Growth regulators (mg/l)				Days required for callus formation after inoculation			Callus formation (%)			Colour of the callus		
BA	NAA	2,4-D	Kn	Leaf	Node	Axial bud	Leaf	Node	Axial bud	Leaf	Node	Axial bud
0.5	-	-	-	24±0.05	15±0.01	13±0.14	48±0.01	53±0.02	64±0.02	WF	WF	LGF
1.0	-	-	-	22±0.05	12±0.06	11±0.01	52±0.12	57±0.08	67±0.04	WF	WF	LGF
1.5	-	-	-	20±0.04	11±0.05	10±0.14	63±0.04	67±0.03	69±0.08	WF	LGF	WF
2.0	-	-	-	23±0.02	14±0.13	12±0.17	67±0.20	72±0.13	78±0.14	GF	GF	GC
2.5	-	-	-	23±0.10	16±0.02	14±0.06	72±0.15	78±0.16	81±0.19	LGF	GC	WF
3.0	-	-	-	24±0.02	17±0.20	15±0.15	64±0.04	73±0.06	74±0.05	LGF	GF	WF
0.5	0.03	-	-	26±0.05	19±0.06	16±0.09	34±0.06	54±0.12	67±0.15	LGF	GC	GF
1.0	0.03	-	-	24±0.25	17±0.31	14±0.12	49±0.16	59±0.07	72±0.04	GC	WF	GF
1.5	0.03	-	-	23±0.04	16±0.05	13±0.01	58±0.14	76±0.11	79±0.14	GF	LGF	GC
2.0	0.03	-	-	22±0.04	16±0.03	14±0.02	76±0.12	82±0.14	85±0.21	WF	GC	GC
2.5	0.03	-	-	21±0.02	14±0.01	8±0.07	49±0.05	69±0.08	67±0.09	GF	GC	LGF
3.0	0.03	-	-	25±0.08	17±0.05	10±0.05	39±0.02	54±0.02	62±0.04	LGF	GC	GF
0.5	-	0.3	-	22±0.04	19±0.05	14±0.01	48±0.04	56±0.01	74±0.04	WF	WF	GC
1.0	-	0.5	-	21±0.01	17±0.1	16±0.15	57±0.05	72±0.06	78±0.02	WF	WF	LGF
1.5	-	1.0	-	20±0.05	15±0.02	12±0.04	75±0.04	78±0.06	83±0.07	GF	LGF	GC
2.0	-	1.5	-	24±0.12	13±0.14	13±0.18	62±0.01	71±0.02	76±0.07	GC	WF	GF
2.5	-	2.0	-	23±0.12	14±0.08	10±0.06	54±0.05	68±0.09	64±0.02	GC	GF	WF
3.0	-	2.5	-	26±0.04	18±0.08	11±0.07	49±0.04	57±0.08	59±0.04	GC	LGF	WF
0.5	-	-	0.1	24±0.05	17±0.04	13±0.02	57±0.01	59±0.03	58±0.05	GC	WF	WF
1.0	-	-	0.3	23±0.01	15±0.08	14±0.06	59±0.05	52±0.06	62±0.04	GC	GF	GF
1.5	-	-	0.5	21±0.12	16±0.20	12±0.16	63±0.01	72±0.02	69±0.02	GF	LGF	GC
2.0	-	-	0.8	20±0.14	14±0.15	10±0.14	72±0.04	76±0.05	79±0.09	GC	WF	LGF
2.5	-	-	1.0	21±0.02	12±0.04	13±0.07	54±0.04	67±0.08	61±0.07	GF	LGF	WF
3.0	-	-	1.5	19±0.12	19±0.04	16±0.09	46±0.01	54±0.05	58±0.04	GC	WF	GF

WF- White coloured, fragile. LGF- Light green coloured ,fragile. GC- Green coloured ,compact. GF- Green coloured ,fragile

MATERIALS AND METHODS

Collection of plant material

Exacum wightianum Arn.(Gentianaceae) was collected during blooming season August, 2010 from the Naduvattam, Uthagamamdalam, the Nilgiri Hills, Western Ghats, Southern India, Tamil Nadu. The plant was identified and authenticated by a plant taxonomist.

Explant source and sterilization

The leaf, nodal and axillary bud segments of *Exacum wightianum* were used as explants. These explants were washed first under running tap water for 30 minutes, then treated with 0.1% (V/V) aqueous solution of Tween-20 (Hi-media, Mumbai) for 15 min, followed by 5 to 6 washes with distilled water thoroughly. Further, the explants were also washed with 0.5% Bavistin for 10 minutes subsequently sterilized with 70% ethanol solution for 5 min followed by sterile distilled water. These explants were again surface sterilized with $HgCl_2$ (0.1% W/V) for 1 min. After repeated rinsing (five times) with distilled water, the explants were ready to inoculate on the culture media. The surface-sterilized explants were aseptically cut into 1-2 cm segments and were carefully inoculated onto the MS culture media (Murashige and Skoog, 1962).

Culture media and culture conditions

The culture media consist of MS salts augmented with 3% (w/v) sucrose and gelled with 0.8% (w/v) agar (Hi-Media, India) .The MS medium is supplemented with combination of 0.1 – 2.0 mg /L BA with 0.05 mg/L NAA, 0.1 – 2.0 mg/L 2, 4-D with 0.05 mg/L NAA and 0.1 – 2.0 mg/L Kinetin with 0.05 mg/L NAA. All the growth regulators were added to the medium before autoclaving and 15ml of medium was dispensed in sterilized culture tubes. The pH of the medium was adjusted to 5.6 to 5.8, followed by autoclaving at 121 °C at 15 psi (1.06 kg/cm2) pressure for 15 to 20 mins. The cultures were incubated at 28 ± 2 °C and 60 µ mol m-2 s-2 light intensity under 12 h photoperiod with cool-white fluorescent tubes (Philips, India) and 55% relative humidity.

RESULTS

In the present study, leaf, node and axial bud explants of *E. wightianum* excised from natural habitat was cultured on MS basal medium supplemented with various concentrations and combinations of BA, BA with NAA, BA with 2,4-D and BA with Kn for the induction of callus.

The leaf, nodal and axial bud explants are cultured on MS basal medium supplemented with BA in different concentrations (0.5, 0.1, 1.5, 2.0, 2.5 and 3.0 mg/ml) and BA in combination with other growth regulators such as NAA with a concentration of 0.03 mg/ml and Kn with various concentrations of 0.1, 0.3, 0.5, 0.8, 1.0 and 1.5 mg/ml respectively. In the above all the combinations, callus formation from leaf, node and axial bud explants were achieved by using MS medium supplemented with BA (2.5 mg/l), BA with NAA (2.0 + 0.03 mg/l), BA with 2,4-D (1.5 + 1.0 mg/l) and BA with Kn (2.0 + 0.8 mg/l) respectively.

The percentage of callus formation was observed in MS basal medium with BA in all explants used. MS medium with BA + NAA combinations showed more per cent Callus formation in the respective explants on MS medium with BA + 2,4-D combinations showed also initiated of callus formation in all the explants. MS basal medium supplemented with BA + Kn combination also showed 72%, 76% and 79% callus formation in the explants of leaf, node and axial bud respectively. Among all the concentrations and combinations of growth regulators, maximum callusing response from axial bud explants were found to be 85%

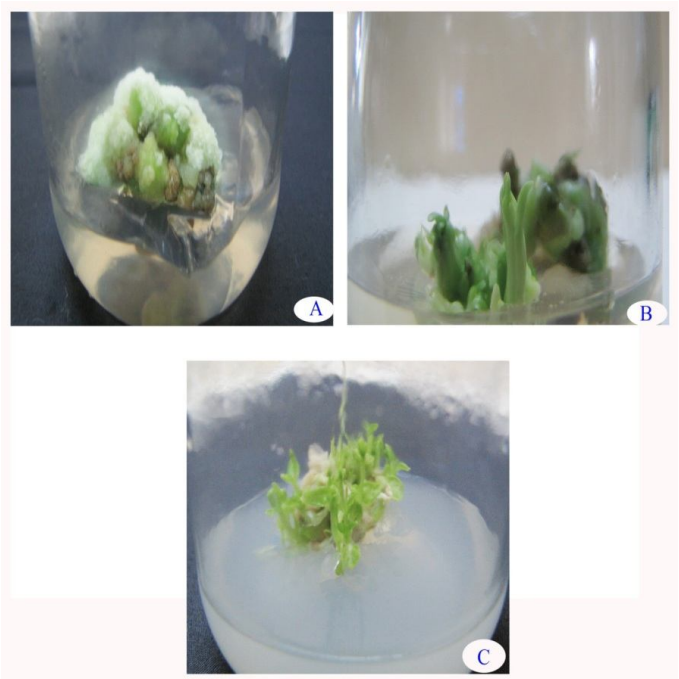

Figure 1: *In vitro* plant regeneration of *Exacum wightianum* axial bud explant. A- Callus induction on MS basal medium supplemented with BA+NAA (2.0+0.03mg/l), B- Subculturing of callus on MS supplemented with BA+NAA(2.0+0.5mg/l), diffrentiated into mulyiple shoots and shoot elangation respectively(C)

Table2. Effect of different concentration of growth regulators on the shoot induction, shoot number and shoot length after subculturing the leaf derived callus of the *Exacum wightianum*

Growth regulators (mg/l)			Shooting response (%)	No. of shoots/callus	Height of Shootlets (cm)
AP	NAA	Kn			
0	-	-	0	0.0 ± 0.0	0.0 ±0.0
0.5	-	-	48±0.02	2.8 ±0.83	0.8 ±0.1
1.0	-	-	60±0.05	6.8 ±0.32	3.4 ±0.14
1.5	-	-	72±0.09	8.2 ±1.0	5.2 ±0.8
2.0	-	-	76±0.04	18.2 ±0.7	9.68 ±0.5
2.5	-	-	84±0.10	21.5 ±0.3	10.26 ±0.3
3.0	-	-	69±0.12	2.6 ±041	0.7 ±0.2
0	0	-	0	0.0 ± 0.0	0.0 ±0.0
0.5	0.3	-	39±0.14	3.4 ±0.5	0.9 ±0.2
1.0	0.3	-	52±0.08	4.9 ±0.2	3.8 ±0.31
1.5	0.3	-	76±0.07	7.8 ±0.6	6.2 ±0.9
2.0	0.5	-	88±0.02	25.1 ±0.1	14.6 ±0.4
2.5	0.5	-	73±0.06	7.2 ±0.4	5.4 ±0.1
3.0	0.5	-	56±0.04	5.8 ±0.7	3.4 ±0.8
0	-	0	0	0.0 ± 0.0	0.0 ±0.0
0.5	-	0.1	49±0.12	2.9 ±0.6	0.6 ±0.1
1.0	-	0.2	58±0.14	4.1 ±0.5	2.9 ±0.4
1.5	-	0.3	71±0.08	7.9 ±0.6	5.3 ±0.2
2.0	-	0.4	78±0.09	14.25 ±0.1	9.47 ±0.3
2.5	-	0.5	65±0.04	10.4 ±0.5	7.9 ±0.1
3.0	-	0.6	52±0.01	8.6 ±0.2	6.2 ±1.0

in MS basal medium with BA+NAA (2.0+0.03 mg/l) (Table.1 and Fig.1).

The various combinations of BA, NAA, 2, 4-D and Kn in basal medium produced different colours of callus. It was observed that the leaf explants cultured in the basal medium containing BA combination with NAA, BA at 3.0 mg/l and BA with NAA at 2.0+0.03 mg/l produced green coloured callus from leaf explants and the nodal explants cultured in the basal medium containing produced green coloured callus. Whereas, MS supplemented with BA in axial bud at the concentration of 2.0 (mg/l) and BA with NAA 2.0+0.03 (mg/l) also produced green coloured callus from the axial bud. It was observed that BA +NAA at the concentrations of 0.2 to 0.03 (mg/l) was best for rapid callus induction and subsequent growth.

Callus grown on MS with BA + NAA possessed high regenerative potential than the other combinations. The stock callus was subcultured on MS containing various concentrations of BA or in Combination with NAA and Kn showed different responses with respect to the number of shoot formation from the various explants. However, the best results were obtained on MS supplemented with BA (2.5 mg/l). The percentage of shoot induction was 84% in BA. Whereas, BA + NAA combinations at the concentrations of 2.0+0.5 mg/l responded more than the other combinations (Table 2).

DISCUSSION

Exacum wightinum is propagated naturally by seeds, but conventional method of propagation cannot meet the requirement, as the number of plants produced is limited. Propagation through seed is hampered by a low germination rate and low viability. On the other hand propagation through *in vitro* approaches offers a scope to propagate plants with desirable traits in larger quantities. MS medium with BA + NAA combinations showed more callus formation in the all explants used. MS medium with BA + 2, 4-D combinations and MS basal medium supplemented with BA + Kn combination also showed more callus formation. The maximum callusing response from axial bud explants were observed in MS basal medium with BA+NAA (2.0+0.03 mg/l). In various concentrations and combinations of BA, NAA, 2, 4-D and Kn in basal medium produced green coloured calli in all explants. Thiem (2003) has reported that callus growth on explant usually interfere with the propagation process. The effects of auxins and cytokinins on shoot multiplication and *in vitro* rooting of various medicinal plants have been reported.

Rajanaika and Krishna (2008) have induced the callus formation and multiple shoots from the stem explants of *Clematis gouriana*, where inoculated on MS medium supplemented with various concentrations of auxins and cytokinins. Callus or callusing of *C. gouriana* was observed from the explants on MS medium containing combinations of BA, NAA, and 2, 4-D. The callus obtained from 2, 4-D supplemented medium failed to produce shoot buds, whereas BA and NAA supplemented medium showed signs of callogenic response and produced callus from the explant. A similar mode of response was observed in the species of *Valeriana edulis* (Castillo *et al*., 2000), *Dioscorea zingiberensis* (Shu *et al.*, 2005), and *Clerodendrum serratum* (Vidya *et al.*, 2005). They sub cultured the callus onto MS medium supplemented with various concentrations and combinations of

auxins and cytokinins with BA + NAA, FAP + IBA, and 2,4-D + FAP. Among these combinations BA with NAA showed an organogenic response and produced only 2 to 3 shoot buds and with 2, 4-D + FAP combinations only callusing was noted, whereas in FAP + IBA combinations there was an organogenic response and shoot buds produced from the callus. Similar results were also reported in several medicinal plants and our findings also accordance with their views in establishing the callus formations.

The results indicated that BA, a cytokinin, played an important role in induction of multiple shoot formation and was very effective in shoot proliferation. However, BA at higher concentrations not only reduced the number of shoots formed but also resulted in stunted growth of the shoots. When the shoot explants of these *in vitro* multiple shoot cultures were transferred onto MS supplemented BA with or without the presence of NAA, more shoots were produced with the presence of NAA in the BA supplemented MS medium. The result showed that the presence of low concentrations of an auxin (NAA) in combination with a cytokinin (BA) positively enhanced the frequency of shoot induction and growth. This indicated the synergistic effect of a cytokinin (BA) and an auxin (NAA). MS medium supplemented with BA and NAA were found to be the most effective medium for shoot multiplication resulting in the formation of an average of shoots per explant. Similar synergistic effects were demonstrated for many *in vitro* propagated plants, for example *Santolina canescens* (Casado *et al*., 2002), *Curcuma zedoaria* and *Zingiber zerumbet* (Christine and Chan, 2007), *Rotula aquatica* (Martin, 2003) and turmeric (Salvi *et al.*, 2002). Pramila Shah *et al.*, (2010) have established an efficient *in vitro* regeneration protocol for *Jatropha curcas* cultured on MS medium without phytohormone showed no visible signs of multiple shoot proliferation. The responding microshoot showed the development of both shoot buds and callus proliferation at the base of explants cultured on MS medium supplemented with different cytokinins. Significantly higher number of shoots was observed on MS media supplemented with BA as compare to kinetin. They observed that few shoots were more in length as compared to other shoots regenerated on multiple shooting media. The BA has often been reported to stimulate shoot proliferation while inhibiting shoot elongation (Figueiredo *et al.*, 2001). BA along with IBA has often been reported to increase the number of shoots in various members of Euphorbiaceae (Shrivastava and Banerjee, 2008).

Our finding is also supported by the previous reports according to which BA had been found more effective over other cytokinins used on multiple shoot regeneration in various plants (Ripley and Preece, 1986). Mohamed khalafalla *et al.*, (2007) have been developed protocol for rapid in vitro propagation by multiple shoot induction of *Vernonia amygdalina*. It is evident from the results that *V. amygdalina* can be easily clonally mass propagated *in vitro* using nodal segments as explants. Propagation from existing meristems yields plants that are genetically identical with the donor plants (Tripathi and Tripathi, 2003). Our results showed that BA alone or in combination with NAA were more effective for shoot multiplication than KIN alone or in combination with 2, 4-D, this mainly because explants grown on the medium containing KIN alone or in combination with 2, 4-D formed excessive callus.

Kannan *et al.*, (2006) have established an *in vitro* propagation of *Excaum travancoricum* (Gentianaceae), an endangered plant of the Southern Western Ghats and is enable to cut off the threat of extinction of this endangered plant. The internodal explants of *E. travancoricum* were cultured on MS with different levels of Thidiazuron or BA showed gradual bulging. Direct shoot morphogenesis was observed on the cut ends of the explants cultured on MS medium with TDZ or BA. TDZ was superior to BA in the induction and proliferation of shoots. Medium with TDZ yielded the higher number of shoots (Vidya *et al*., 2005).

CONCLUSION

It's concluded that indirect *in vitro* protocol was developed for *E. wightianum* which could be able to produce a large number of plant lets. It may be useful to offer a potential *in vitro* method for conservation and establishment in various localities. The highest degree of callus proliferation was found on MS basal medium with BA and MS medium with BA + NAA combinations in the respective explants were found to be the best. Among all the concentrations and combinations of growth regulators, maximum callusing response from axial bud explants was observed. The percentage of shoot induction was 84% in BA and BA + NAA combinations responded more than the other combinations. Protocol described herein could be used for the commercial establishment of *E. wightianum.*

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