

A SIMPLE AND EFFICIENT MICROPROPAGATION PROTOCOL FOR DEVELOPING PLANTLETS OF *EXACUM BICOLOR* ROXB. – AN ENDANGERED, ORNAMENTAL, AND ANTIDIABETIC HERB

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ABSTRACT

Objective: *Exacum bicolor* Roxb. is an endangered medicinal herb due to overexploitation by humans and its inefficient vegetative reproduction. Here, we report an efficient and simple procedure for the regeneration of *E. bicolor* Roxb. using leaf as an explant.

Methods: The optimal concentrations of the hormones needed for callus induction were determined by full factorial method using DOE (Design expert ver. 8.0). The hormones selected based on literature were kinetin, indole acetic acid, and 6-Benzylaminopurine (BAP). Multiple shoot regeneration was carried out in liquid and solid media with the optimal concentrations of the hormones obtained by DOE. Rooting was initiated using Murashige and Skoog media containing naphthalene acetic acid 0.5 mg/l, indole butyric acid (IBA) 1.0 mg/l, and gibberellic acid 3 0.5 mg/l along with 0.2% of activated charcoal.

Results: Analysis of full factorial design run showed that BAP in combination with kinetin was effective for the growth of callus and multiple shoot regeneration was higher in liquid media (81.25%). The rate of rooting was observed to be 88.23% and the average number of roots was 0.26. Plantlets with budding apical region and well-established leaves and roots were observed in 30 days.

Conclusion: The protocol reported here can be used for effective production of *E. bicolor* plants in a shorter duration compared to the conventional approach.

Keywords: *Exacum*, DOE, Regeneration of plantlets.

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INTRODUCTION

Plants of genera *Exacum* (Gentianaceae) are well known for their ornamental [1,2] and medicinal properties [3-5]. These plants are mostly found in the temperate and subtropical regions and include about 1738 species. So far, around 153 species from 17 genera have been identified in India [6]. The plant grows to a height of 25–120 cm and its vegetative phase is from June to August with a flowering period from September to November [7,8].

In recent times, there has been a rapid decline of its population due to loss of its habitat. The approach currently being used to increase their population is by seed germination. However, it is difficult to reproduce these plants due to the high rate of seed dormancy [7,9,10].

Hence, plant tissue culture is a viable option for increasing its population. Many *in vitro* culture protocols have been developed from nodal regions of the plant [1-3,11,12]. However, it has been observed that micropropagation by these methods is elaborate, not highly successful and time consuming.

Here, we report a simple and efficient protocol for developing plantlets from leaf explants. Full factorial design was used to predict the optimal concentrations of the hormones for obtaining a well-developed callus within 30 days. The leaves from a well-grown healthy flowering plant were used as explant for developing plantlets. Further, these plantlets were grown on shooting and rooting media to obtain a complete plant.

METHODS

The Murashige and Skoog (MS) media and the hormones naphthalene acetic acid (NAA), IBA, BAP, and kinetin were procured from HiMedia, India, and gibberellic acid 3 (GA3) was procured from LOBA Chemie, India.

Preparation of explants and shoot bud initiation

The plant *Exacum bicolor* was obtained from the hilly regions of Sakaleshpura (Bisele ghat) and authenticated by NADRI-Ministry of AYUSH in Bengaluru-India (Fig. 1). It was collected in early September when the flowers had just bloomed and new buds were developing in the apical region of the plant. The young and tender leaves of the plant were used as explant in our studies.

Surface sterilization was performed as per the standard protocol given by Bhojwani and Razdan [13]. The leaf explants of 0.5×0.5 cm were inoculated into 25 ml of MS media supplemented with plant growth regulators.

Factorial design of 2³ (Design expert Ver. 8.0) was used to obtain the optimal concentrations of the plant hormones for maximal callus production. Three hormones, namely, BAP, indole acetic acid (IAA), and kinetin were selected at two levels (high and low values) based on available literature [3,7,8,14].

The experiments were conducted as per the DOE generated (Table 1) for the three hormones at two levels, namely, BAP (0.1–2.5 mg/l), IAA (0.0–0.5 mg/l), and kinetin (0.0–3.0 mg/l). The response was considered with respect to weight of callus and growth in days. ANOVA was used to determine the significance of the factors. The leaf explants were inoculated into the tissue culture flasks as per DOE and incubated at 24±2°C under a 16/8 h (light/dark cycle) photoperiod provided with cool white fluorescent light (1500–3000 Lux) for 30 days.

Shoot proliferation

A well-developed greenish rounded callus (1 g) obtained from the explants was used for shoot proliferation. The callus obtained was dissected aseptically (1.0–1.5 cm) and reinoculated into MS media containing BAP 10 µM and kinetin 2 µM to develop florets as per the

Table 1: Factorial design of 2³ DOE generated using Design expert Ver. 8.0

Std.	Run	Factor 1 A: BAP mg/l	Factor 2 B: IAA mg/l	Factor 3 C: Kinetin mg/l	Response 1 growth in days	Response 2 weight of callus
1	1	0.10	0.00	0.00		
6	2	2.50	0.00	3.00		
4	3	2.50	0.50	0.00		
5	4	0.10	0.00	3.00		
8	5	2.50	0.50	3.00		
3	6	0.10	0.50	0.00		
2	7	2.50	0.00	0.00		
7	8	0.10	0.50	3.00		

Table 2: ANOVA for response with respect to growth

Response 1 growth ANOVA for selected factorial model analysis of variance table (partial sum of squares - Type III)					
Source	Sum of squares	df	Mean square	F Value	p-value Prob > F
Model	165.75	6	27.63	221.00	0.0514 not significant
A-BAP	0.13	1	0.13	1.00	0.5000
B-IAA	28.13	1	28.13	225.00	0.0424
C-Kinetin	21.13	1	21.13	169.00	0.0489
AB	55.13	1	55.13	441.00	0.0303
AC	55.12	1	55.12	441.00	0.0303
BC	6.13	1	6.13	49.00	0.0903
Residual	0.12	1	0.12		
Cor Total	165.88	7			

The model F-value of 221.00 implies that there is a 5.14% chance that a "Model F-Value" This large could occur due to noise. Values of "Prob > F" < 0.0500 indicate model terms are significant. In this case, B, C, AB, and AC are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model

Std. Dev.	0.35	R-Squared	0.9992
Mean	19.38	Adj R-Squared	0.9947
C.V. %	1.82	Pred R-Squared	0.9518
PRESS	8.00	Adeq Precision	33.261

The "Pred R-Squared" of 0.9518 is in reasonable agreement with the "Adj R-Squared" of 0.9947. "Adeq Precision" measures the signal-to-noise ratio. A ratio greater than 4 is desirable. Your ratio of 33.261 indicates an adequate signal. This model can be used to navigate the design space

Coefficient	standard	95% CI	95% CI	VIF		
Factor	Estimate	Df	Error	Low	High	VIF
Intercept	19.38	1	0.12	17.79	20.96	
A-BAP	0.12	1	0.12	-1.46	1.71	1.00
B-IAA	1.87	1	0.12	0.29	3.46	1.00
C-Kinetin	-1.63	1	0.12	-3.21	-0.037	1.00
AB	2.63	1	1	1.04	4.21	1.00
AC	2.63	1	1	1.04	4.21	1.00
BC	0.88	1	1	-0.71	2.46	1.00

Final equation in terms of coded factors: Growth = +19.38+0.12*A+1.87*B-1.63*C+2.63*A*B+2.63*A*C+0.88*B*C

Final equation in terms of actual factors: Growth = +25.55208-4.27083*BAP-7.37500*IAA-3.56250*Kinetin+8.75000*BAP*IAA+1.45833*BAP*Kinetin+2.33333*IAA*Kinetin

protocol given by Appaji *et al.* [12]. They were incubated at 24±2°C under a 16/8 h (light/dark cycle) photoperiod provided with cool white fluorescent light (1500-3000 Lux).

After incubation, the percentage of shoot induction, time taken for bud initiation (marked by a separation layer on the edges of the petiole), and the growth rate of the buds were recorded.

Table 3: ANOVA for response with respect to weight of callus

Response 2 weight of callus ANOVA for selected factorial model analysis of variance table (partial sum of squares - Type III)						
Source	Sum of squares	df	Mean square	F Value	p-value Value Prob > F	
Model	0.10	6	0.017	10.42	4.76	0.2736
A-BAP	7.744E-003	1	7.744	E-003	0.1139	
B-IAA	0.050	1	0.050	30.57	5.85	0.2496
C-Kinetin	9.515E-003	1	9.515	E-003	3.33	0.3193
AB	5.413E-003	1	5.413	E-003	0.024	0.9031
AC	3.828E-005	1	3.828	E-005	0.1475	
BC	0.029	1	0.029	17.97		
Residual	1.627E-003	1	1.627	E-003		
Cor total	0.10	7				

The "Model F-value" of 10.42 implies the model is not significant relative to the noise. There is 23.28% chance that a "Model F-value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case, there are no significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model

Std. Dev.	0.040	R-Squared	0.9843
Mean	0.61	Adj R-Squared	0.8898
C.V. %	6.62	Pred	0.0079
		R-Squared	
PRESS	0.10	Adeq Precision	8.917

A negative "Pred R-Squared" implies that the overall mean is a better predictor of your response than the current model. "Adeq Precision" measures the signal-to-noise ratio. A ratio greater than 4 is desirable. Your ratio of 8.917 indicates an adequate signal. This model can be used to navigate the design space

Coefficient	Standard	95% CI	95% CI	VIF		
Factor	Estimate	df	Error	Low	High	VIF
Intercept	0.61	1	0.014	0.43	0.79	
A-BAP	0.031	1	0.014	-0.15	0.21	1.00
B-IAA	-0.079	1	0.014	-0.26	0.10	1.00
C-Kinetin	0.034	1	0.014	-0.15	0.22	1.00
AB	-0.026	1	0.014	-0.21	0.16	1.00
AC	2.188E-003	1	0.014	-0.18	0.18	1.00
BC	0.060	1	0.014	-0.12	0.24	1.00

Final equation in terms of coded factors: Weight of callus = +0.61+0.031*A-0.079*B+0.034*C-0.026*A*B+2.188E-003*A*C+0.060*B*C

Final equation in terms of actual factors: Weight of callus = +0.65468+0.045781*BAP-0.44458*IAA-0.018897*Kinetin-0.086708*BAP*IAA+1.21528E-003*BAP*Kinetin+0.16123*IAA*Kinetin

The florets developed were reintroduced into both solid and liquid media of the same composition incubated. The multiplication rate and number of new shoots per explant which were greater than 0.5 cm were recorded.



Fig. 1: Authentication of the plant by NADRI-Ministry of AYUSH in Bengaluru, India

Rooting

Individual shoots (>1.2 cm height) obtained from liquid culture were cut off and transferred into rooting media, namely, MS media containing NAA 0.5 mg/l, IBA 1.0 mg/l, and GA3 0.5 mg/l and 0.2% of activated charcoal. The plantlets were incubated at 24±2°C under a 16/8 h (light/dark cycle) photoperiod provided with cool white fluorescent light (1500–3000 Lux).

The percentage of root induction, root numbers, and the growth rate of roots were observed and recorded. Observations were recorded at regular intervals of 48 h.

The induction rate, multiplication, and rooting rate were calculated as given below.

Induction rate (%) = The number of induced explants/the number of total initial explants × 100%.

Multiplication rate (%) = The total number of buds (≥0.3 cm)/the number of initial buds on the sub cultured explants × 100%.

Rooting rate (%) = The number of the rooted plantlets/the number of total shoots × 100%.

Average number of roots = The total number of roots/the number of rooted seedlings.

RESULTS AND DISCUSSION

Preparation of explants and shoot bud initiation

The young tender leaves of the plant were used as explants. Factorial design of 2³ (Design expert Ver. 8.0) was used to obtain the optimal concentrations of the plant hormones for maximal callus production. The experiments were conducted as per the DOE generated for the three hormones at two levels, namely, BAP (0.1–2.5 mg/l), IAA (0.0–0.5 mg/l), and kinetin (0.0–3.0 mg/l) and the response was considered with respect to weight of callus and growth in days. The ANOVA with respect to growth and weight of

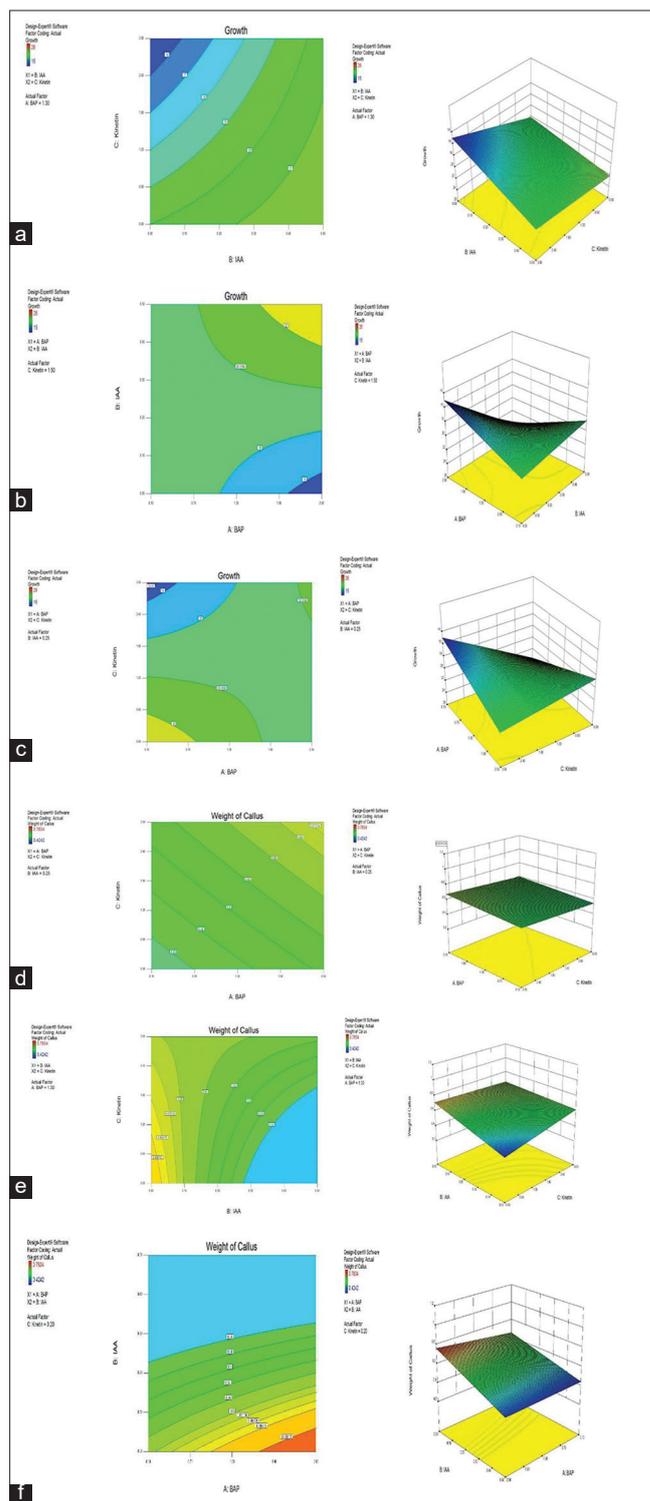


Fig. 2: (a) Contour plots of growth response with KINETIN and IAA. (b) Contour plots of growth response with BAP and IAA. (c) Contour plots of growth response with BAP and kinetin. (d) Contour plots of weight of callus with BAP and kinetin. (e) Contour plots of weight of callus with IAA and kinetin. (f) Contour plots of weight of callus with IAA and BAP

callus is shown in Tables 2 and 3, respectively. The contour plots of the responses are shown in Fig. 2a-f. Healthy greenish callus was formed from the explants within 40 days. It was observed that BAP in combination with kinetin was effective for the rapid growth of callus (Fig. 3). Hence, these concentrations were considered for all further studies.



Fig. 3: Callus developed from leaf explants



Fig. 4: Florets developed in solid and liquid media from the callus in 20 days



Fig. 5: Plantlets bearing roots grown in the rooting medium with activated charcoal

Shoot proliferation

A well-developed greenish rounded callus (1 g) obtained from the explants was used in shoot proliferation. The induction rate of the explants was 500% and shoot proliferation rate was 75%. The time taken for bud proliferation was 60 days.

The florets developed were reintroduced into both solid and liquid media of the same composition and incubated. After 15 days, better plantlet formations were observed in liquid media compared to solid media. The florets formed were well developed with distinct nodal regions and leaves at each node. The multiplication rate was 81.25% in liquid media.

Rooting

Individual shoots (≥ 1.2 cm height) obtained from liquid culture were cut off and transferred into rooting media (Fig. 4). The initial growth

of roots from the base of the plantlets was observed within 3 weeks. Twenty-four plantlets with well-established roots and green leaves at every node with a budding apical region were observed within 30 days (Fig. 5). The rate of rooting was 88.23% and the average number of roots was 0.26.

CONCLUSION

Many studies have reported the conventional method for micropropagation of plants belonging to this genus [2,3,14-17]. Obtaining the plantlets by the conventional method of propagation involves the usage of nodal regions as explants [11,12] and the duration required to obtain plantlets varies from 90 days to 112 days. Here, we report a modified protocol which can produce plantlets with well-established roots in 90-110 days.

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AUTHORS' CONTRIBUTIONS

S.S.A. has performed the experiments. S.V. has supervised the study. Both S.S.A. and S.V. have contributed to the final version of the manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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