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Optimization of Media Constituents for Shoot Regeneration from Leaf Callus Cultures of *Decalepis hamiltonii* Wight. & Arn.

Jacob George, Harsh Pal Bais, and G.A. Ravishankar[1]
Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore 570 013, India

P. Manilal
Planning, Monitoring and Coordination Department, Central Food Technological Research Institute, Mysore 570 013, India

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Abstract. **Response surface methodology was utilized in statistical optimization of three quality factors (the number of multiple shoots, shoot length, and number of leaves) pertaining to regeneration of plantlets from leaf calli of *Decalepis hamiltonii* Wight. & Arn. (swallow root). The variables evaluated were the levels of sucrose, BA, and NAA each at two different concentrations. Response surfaces for shoot length and multiple shoot number were useful in achieving optimal levels of media constituents and in understanding their interactions, but response surfaces for number of leaves were not. The data indicate that sucrose, BA, and NAA levels may be manipulated to increase or decrease quality factors chosen. This approach may be useful in developing a micropropagation protocol for *D. hamiltonii*. Chemical names used: benzyladenine (BA); napthaleneacetic acid (NAA).**

Response surface methodology (RSM) is an analytical tool to determine the optimum conditions for a multivariable system and has been applied for optimizing media for tissue culture (Myers, 1971). Both nutritional and hormonal factors play a crucial role in the growth and development of plants in vitro (George, 1996). The level of sucrose is a major factor, as are the nature, concentrations, and ratios of various endogenous and exogenous auxins and cytokinins.

In such studies, RSM (Dziezak, 1990) has been used to optimize the combinations of growth regulators and stage of maturity of fruit for callus initiation and development in *Psidium guajava* L. (Madhavi et al., 1992). RSM was used to analyze the interaction of hormones and polyamines in affecting the plating efficiency of protoplasts of *Ipomoea batatas* (L.) Poir. (Eiler et al., 1988), and to optimize the immobilization conditions for higher yields of capsaicinoids in *Capsicum frutescens* L. (Suvarnalatha et al., 1993).

Decalepis hamiltonii Wight. & Arn. is a climbing shrub distributed in the Deccan Peninsula and Western Ghats of peninsular India (Santapau and Henry, 1973). Of late, the highly aromatic roots of this plant have been subjected to over exploitation by destructive harvests, endangering its survival in its wild habitat. Moreover, the absence of any organized cultivation of the plant (M. Sanjappa, personal communication, Botanical Survey of India, Calcutta) calls for immediate conservation measures.

In the present study, in vitro growth and development of multiple shoots from leaf explants of *Decalepis hamiltonii* was studied (Fig. 1) using RSM to optimize the concentrations of various factors affecting the initiation of multiple shoots. This paper reports the first use of RSM to study the effects of BA, NAA, and sucrose in multiple shoot induction and to develop an efficient micropropagation system for *Decalepis hamiltonii*.

Materials and Methods

Source of explants. Healthy plants of *Decalepis hamiltonii* were collected from Gumballi forest ranges in BR hills, Mysore District, India.

Media and culture conditions. The medium was supplemented with BA and NAA for *D. hamiltonii* at prescribed concentrations as per the experimental design. The media (15 mL) were prepared in test tubes (60-mL capacity) and the pH adjusted to 5.8 ± 0.1. Agar-agar (HiMedia, Mumbai, India) was added at 0.8% (w/v) as the gelling agent and homogenized by boiling, and the media were subsequently autoclaved at 1.2 $kg \cdot cm^{-2}$ for 20 min. Leaf segments measuring 5–10 mm were surface-sterilized with mercuric chloride (0.15% w/v) and transferred to the medium under aseptic conditions in a laminar airflow chamber. The cultures were maintained at a constant 25 ± 2 °C under an 18-h photoperiod (4.4117 $J \cdot m^{-2} \cdot s^{-1}$) provided by cool-white fluorescent lamps (four tubes, 122 cm, 40 W each, Phillips, India). Data on multiple shoot induction were recorded after 30 d of culture.

Multiple shoots were initiated from callus derived from the explants. After 30 d of culture the shoot cultures from the eight replicates of each treatment were analyzed for the number of shoots per explant, shoot length, and leaf number. The data obtained were subjected to Fisher's F test and (estimated coefficient of significance, $P \leq 0.05$) tested using Student's *t* test (Steele and Torrie, 1980).

The response surface graphs were obtained from the regression equation, keeping the response function on the Z axis; the X and Y axes represent two independent variables, while the other variable is kept at a constant level.

Experimental design. An eight-point, full, two-way factorial design was used with three center points (i.e., three replicates used for estimation of variance) (Box et al., 1978; Nabais and Malcata, 1995) aimed at fitting the polynomial of the following form:

$$y = b_o + \sum b_i x_i + \sum b_{ij} x_i x_j,$$
$$i,j = 1,n,\ i \neq j,$$

where b_o is the intercept, b_i ($i = 1,2,3$) are

Fig. 1. Shoot regeneration from leaf calli of *Decalepis hamiltonii.*

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[1]To whom reprint requests should be addressed. Fax: +91-821-517233; E-mail: pcbt@cscftri.ren.nic.in

Table 1. Response surface design for 2 × 2 × 2 factorial, plus three replicates of the centerpoint, giving observed (O^b) and predicted (P^a) values of the response for the fitted model.

Expt.	Sucrose (%)	NAA ($mg·L^{-1}$)	BA ($mg·L^{-1}$)	Coded factors			Shoot length (cm)		Shoot no.	
no.	X_1	X_2	X_3	Z1	Z2	Z3	P^a	O^b	P^a	O^b
1	1	0.05	0.05	–1	–1	–1	5.452	5.58	2.3	2
2	3	0.05	0.05	1	–1	–1	5.972	5.84	2.3	2.6
3	1	1.9	0.05	–1	1	–1	4.672	4.5	1.7	1.8
4	3	1.9	0.05	1	1	–1	4.152	4.32	1.7	1.6
5	1	0.05	1.9	–1	–1	1	6.474	6.26	4.0	3.6
6	3	0.05	1.9	1	–1	1	6.994	7.2	4.0	4.4
7	1	1.9	1.9	–1	1	1	5.122	5.24	2.6	2.6
8	3	1.9	1.9	1	1	1	4.602	4.68	2.6	2.6
9	2	0.97	0.97	0	0	0	5.43	4.48	2.65	2
10	2	0.97	0.97	0	0	0	5.43	4.48	2.65	2.2
11	2	0.97	0.97	0	0	0	5.43	4.54	2.65	2

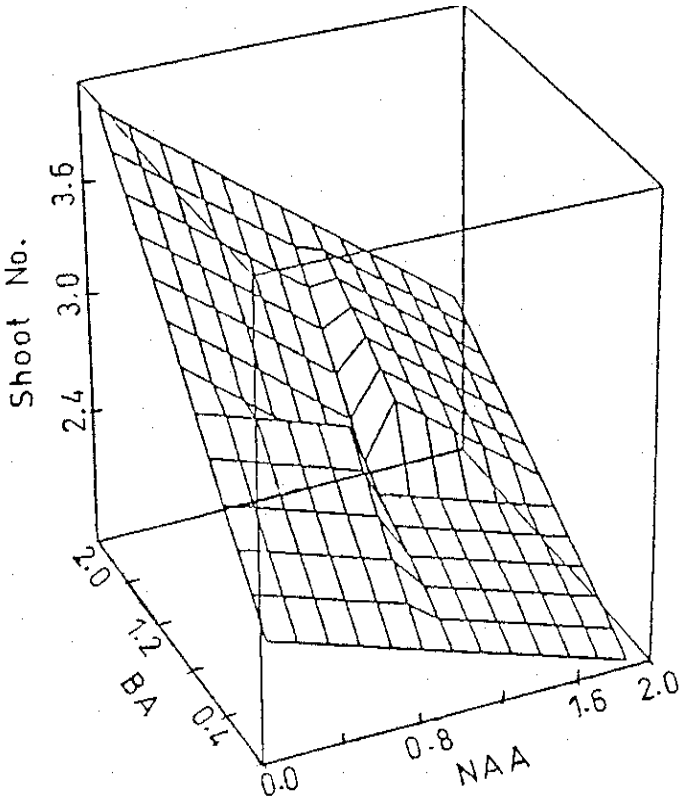

Fig. 2. Surface plot of shoot number in *D. hamiltonii* with variable concentrations of NAA and BA.

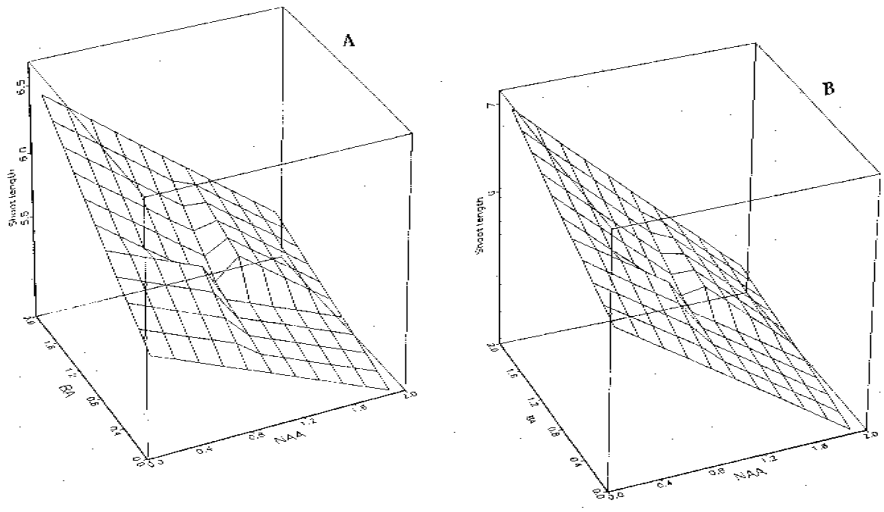

Fig. 3. Surface plot of shoot length in *D. hamiltonii* as affected by concentration of NAA and BA in media containing (**A**) 1% sucrose or (**B**) 3% sucrose.

parameters associated with the linear terms in the model, b_{ij} (i,j = 1,2,3) are parameters associated with the cross-product terms, and n is the number of factors involved. The model was fitted using standard response surface methodology.

Three critical parameters known to influence the initiation and development of multiple shoots in *D. hamiltonii* were selected using two levels of each. The results obtained for the parameters are tabulated in Table 1. The model was designed with the assumption that initiation of multiple shoots from calli obtained from the leaf explants and their subsequent development are functions of its interacting factors, such as sucrose, BA, and NAA. The significance of the coefficients in the polynomial was tested against tabulated values (*t* test) as suggested by Akhanozarova and Kafarov (1978) and Fisher's F test for the validation of the model.

Optimization of the function was estimated using MS-X (Microsoft Corp., Redmond, Wash.) ver. 5.0 Solver modules. The maximizing steps with the constraints placed as the lowest and a highest coded value for each of the variables was carried out using a quasi-Newton search algorithm.

Results and Discussion

The significance of the coefficients in the second-order polynomial was tested against the tabulated values using the *t* test after validating the model (Akhanozarova and Kafarov, 1978). Increasing the concentration of NAA reduced shoot number (Fig. 2) while sucrose had no effect (data not shown). Maximum response was obtained when the NAA concentration was least (0.05 $mg·L^{-1}$) and the BA was maximum (1.90 $mg·L^{-1}$). Both responses were linear. The fitted model for the shoot number response is:

$y = 2.65 - 0.5\ X_2 + 0.65\ X_3 - 0.2\ X_2X_3$,

where X_2 is the NAA level in $mg·L^{-1}$, X_3 is the BA level in $mg·L^{-1}$, and y is the predicted shoot number. For evaluating shoot length, the fitted model for the response is:

$y = 5.43 - 0.793\ X_2 + 0.368\ X_3 - 0.268\ X_1X_2 - 0.143\ X_2X_3$,

where X_2 is the NAA level in $mg·L^{-1}$, X_3 is the BA level in $mg·L^{-1}$, and y is the predicted shoot length.

In Fig. 3 A and B the concentration of sucrose was kept constant (lowest and highest,

Table 2. Decision table showing optimum conditions for shoot length.

Sl. no.	Sucrose (%) (X_1)	NAA (mg·L^{-1}) (X_2)	BA (mg·L^{-1}) (X_3)	Maximum shoot length (cm)
(i)	1	0.05	1.9	6.47
(ii)	3	0.05	1.9	6.99
(iii)	3	0.05	0.05	5.97
(iv)	1	1.9	1.9	5.12

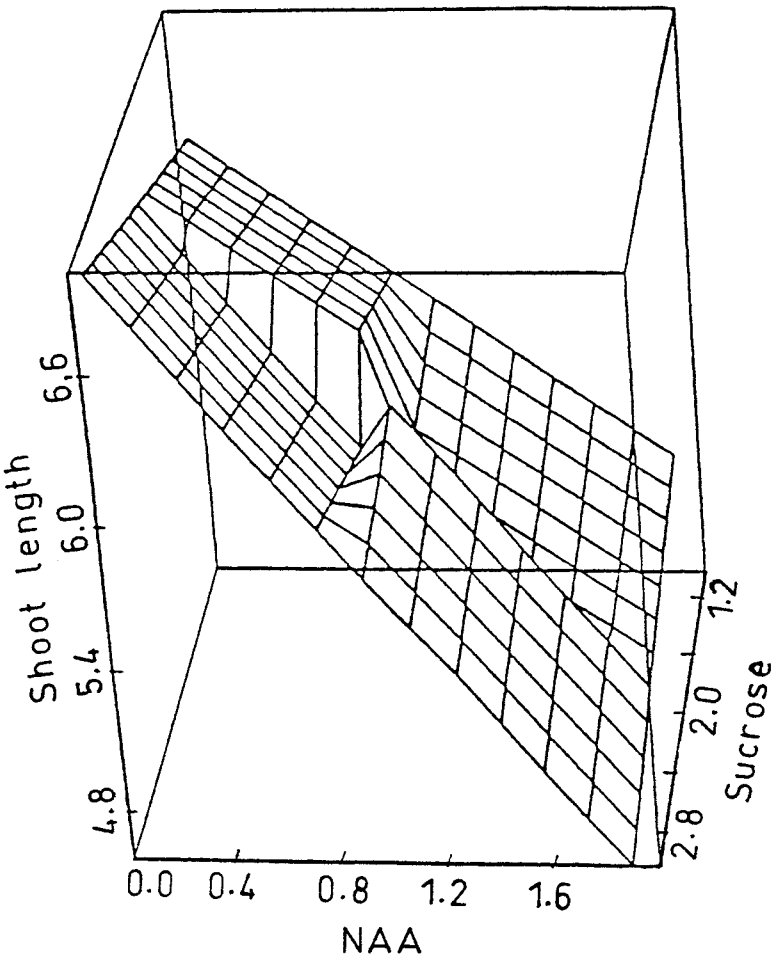

Fig. 4. Surface plot of shoot length in *D. hamiltonii* with maximum concentration of BAP (1.9 mg·L^{-1}).

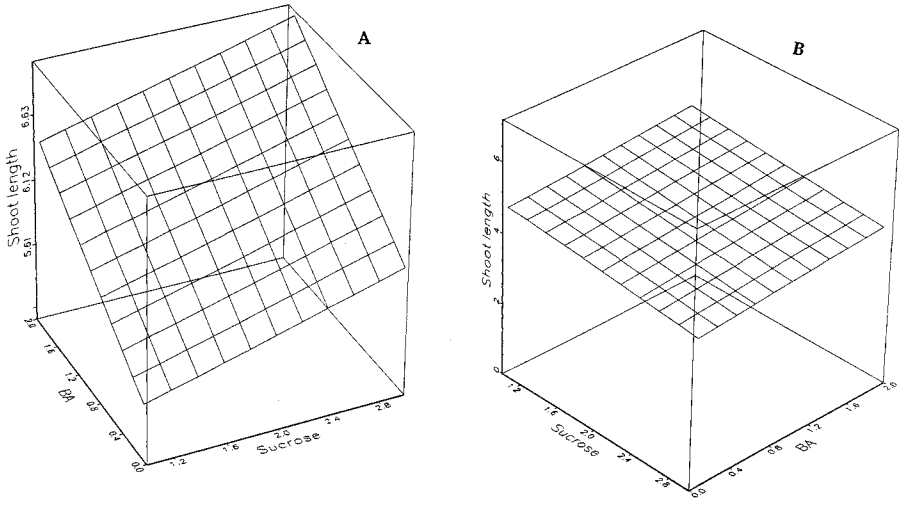

Fig. 5. Surface plot of shoot length in *D. hamiltonii* as affected by concentrations of BA and sucrose in media containing NAA at (**A**) 0.05 mg·L^{-1} or (**B**) 1.9 mg·L^{-1}.

respectively) while the other two factors varied. When the sucrose concentration was least, the maximum shoot length was 6.474 cm; when the concentration was highest, maximum shoot length was 6.994 cm. In both cases, as the equations indicate, a lower concentration of NAA coupled with a higher concentration of BAP favored increased shoot numbers (Table 2). Thus, higher sucrose levels (3%) together with maximum BA (1.9 mg·L^{-1}) and minimum NAA (0.05 mg·L^{-1}) resulted in maximum shoot length. One may infer that the interaction of sucrose with NAA was the decisive factor that determined shoot length.

When BAP levels were maximal and other factors varied, maximum shoot length was obtained when the concentrations of NAA and BA were 0.05 and 1.9 mg·L^{-1}, respectively (Fig. 4). Irrespective of varying conditions, the predictive values were in the range of 5–7.

When NAA level was lowest and other factors were variable, i.e., increased sucrose concentration at 3% and fixed value of BA at 0.05 mg·L^{-1}, the range of predictive values was 5.5 to 7 (Fig. 5A).

When NAA level was highest and the other factors were variable, maximum shoot length was 5.122 cm with minimal sucrose and maximal BA concentrations (Fig. 5B).

The optimum combination of concentrations of BA and NAA was 1.9 and 0.05 mg·L^{-1}, respectively. Shoot number exhibited a positive linear relationship with BA concentration and was maximum at the highest levels of BA. However, the increase in shoot number was evident in clusters of short shoots. Maximum shoot length was obtained when sucrose concentration was 3%, NAA concentration minimum (0.05 mg·L^{-1}), and BA concentration maximum (1.9 mg·L^{-1}) (Table 3). This showed the positive effect of BA on shoot length and shoot multiplication, and justifies exclusion of NAA in later stages. However, the same concentrations of the two hormones and sucrose did not effect the number of leaves.

Our emphasis on the number and length of shoots developed from the leaf callus is of significance, since a good multiplication rate is the most important factor in rendering a propagation protocol cost-effective. The optimum combination of factors obtained by using the methodology has been effective in developing an efficient micropropagation protocol for *D. hamiltonii*.

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Table 3. Effect of concentration of constant parameter and varying factors with observed response range (shoot length).

Constant	Concn ($mg \cdot L^{-1}$)	Varying factors	Observed response range (cm)	Percentage increase in shoot length of highest means minus lowest observed value
Sucrose	10,000	NAA, BA	5.12–6.47	26.4
Sucrose	30,000	NAA, BA	4.60–6.99	52
BA	1.9	Sucrose, NAA	4.60–6.99	52
NAA	0.05	Sucrose, BA	5.45–6.99	28.3
NAA	1.9	Sucrose, BA	4.15–5.12	23.4

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