In Vitro Cell.Dev.Biol.—Plant (2012) 48:92–98
DOI 10.1007/s11627-011-9394-z

Statistical optimization of media for enhanced azadirachtin production from redifferentiated zygotic embryo cultures of neem (*Azadirachta indica* A. Juss.)

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Received: 10 January 2011 / Accepted: 11 August 2011 / Published online: 23 September 2011 / Editor: J. Finer

Abstract Callus cultures from zygotic embryos of neem (*Azadirachta indica*) were initiated and analyzed for azadirachtin production. Medium components were screened and optimized using the statistical techniques of Plackett–Burman and response surface methodology. The Plackett–Burman design, with five medium components (Murashige and Skoog major salts, sucrose, casein hydrolysate, indole-3-acetic acid, and N^6-benzylaminopurine), was performed to screen the variables that significantly affected azadirachtin production. The three variables—Murashige and Skoog major salts, sucrose, and N^6-benzylaminopurine—significantly affected azadirachtin production and were significant factors for optimization using response surface methodology. The experimental results were fitted to a second-order polynomial model with a correlation coefficient (R^2) of 0.9582. The optimal concentrations of variables for maximum azadirachtin production were full-strength Murashige and Skoog major salts, 5.68% sucrose, and 10.42 μM N^6-benzylaminopurine. The maximum azadirachtin production by the predicted model was 5.13 mg/g dry weight, which was in agreement with the actual experimental value of 4.97 mg/g dry weight.

Keywords Azadirachtin · Neem · Optimization · Plackett–Burman · Response surface methodology (RSM)

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Introduction

Azadirachta indica A. Juss., commonly known as neem, is a multipurpose evergreen tree in the mahogany family, Meliaceae. It is a native of India (Koul *et al.* 1990) and has spread to many countries. Its various parts, particularly the leaves, the bark, and the seeds, possess multifaceted therapeutic, agrochemical, and economic uses. The broad-spectrum properties of neem can be ascribed to the numerous secondary metabolites present in the genus. Among them, azadirachtin is one of the most important compounds obtained from neem seed kernels (National Research Council 1992).

Azadirachtin ($C_{35}H_{44}O_{16}$), a tetranortriterpenoid, possesses powerful antifeedant, growth regulatory, and antifertility effects against a broad spectrum of insects. Apart from this, the compound has largely been found responsible for diverse types of bioactivities of neem, including antimicrobial, antifungal, and antiviral properties. The demand for azadirachtin results from its immediate application as an eco-friendly, biodegradable, and nontoxic pesticide. Neem products performed equally or sometimes better than neurotoxic, broad-spectrum synthetic pesticides like pirimiphos-methyl (Actellic 25 EC), permethrin, and lindane (γ-BHC; Ogunwolu and Oddunlami 1996; Lale and Mustapha 2000). Besides its properties as a biopesticide, azadirachtin can also inhibit the sexual development of malarial parasites (Jones *et al*. 1994). To date, the only commercially feasible way to produce azadirachtin is extraction from seeds, but this approach is disadvantageous because of the variability in yield resulting from parent

plant age/maturity, heterozygosity, and other environmental/physiological factors (Ermel *et al*. 1983, 1987; Benge 1989; Ketkar *et al*. 1993; Ermel 1995). Moreover, neem trees flower once a year, and only about one third of the fruits are collected as the pericarp of a fully ripe fruit contains high levels of carbohydrates which ferment during improper handling and storage (Jayaraj *et al*. 1993; Vyas and Mistry 1996; Venkateswarlu and Mukhopadhyay 1999). Furthermore, the chemical synthesis of azadirachtin is very difficult because of its highly complex structures and the specific stereochemical requirements. Hence, the development of a viable method for azadirachtin production is needed. In this context, plant cell and organ cultures offer an attractive alternative to whole plant extraction for homogeneous, controlled production. *In vitro* techniques offer a consistent yield and high-quality azadirachtin production, regardless of the season and the regions.

Realizing the immense potential of azadirachtin, it is worthwhile to optimize its production for maximum yields. Therefore, attempts were made in the present study to enhance its production by developing processes that are economically viable. The conventional methods for optimization of the medium involve varying "one-factor-at-a-time" while keeping the others constant. Unfortunately, this approach cannot detect interactions among the various optimized factors. Moreover, these methods are laborious, time-consuming, and impractical. To overcome this difficulty and to evaluate and understand the interactions between different physiological and nutritional parameters, response surface methodology (RSM) has been widely used (Houng *et al*. 1989; Yalimaki *et al*. 1991; Sunita *et al*. 1998). RSM integrates the interaction of various parameters, generally resulting in higher production yields and limiting the number of experiments. In addition to analyzing the effects of independent variables, this experimental methodology generates a mathematical model that accurately describes the overall process.

We report here the use of a statistical approach called Plackett–Burman (PB) design which ignores the interactions among factors. Only the most effective factors with high significance levels are selected for further optimization, while those with lower significance levels or with small effects on the response value are omitted in further experiments. Following this, the central composite design (CCD) falling under the RSM is adopted to determine the relationship between factors and responses.

For the PB design, media components were chosen on the basis of our previous study (data not shown) in which calluses from various explants of neem (zygotic embryo, leaf, anther, and ovary) were established and screened on the best culture medium for azadirachtin production. Analysis of these *in vitro* cell lines showed the presence of azadirachtin in all the samples tested. However, the amount varied with the media composition and the cell differentiation response. We observed that the medium supporting organogenesis also supported higher azadirachtin biosynthesis. Among all the *in vitro* cell lines (four dedifferentiated and four redifferentiated lines), the highest azadirachtin yield (2.33 mg/g dry weight, DW) was observed in redifferentiated callus, which originated from early dicotyledonary zygotic embryos. An elite cell line showing shoot organogenesis was proliferated on Murashige and Skoog (MS) medium supplemented with 9.0 μM N^6-benzylaminopurine (BAP), 5.0 μM indole-3-acetic acid (IAA), and 500 mg/l casein hydrolysate (CH). On the other hand, dedifferentiated callus of zygotic embryo that proliferated on 0.5 μM 2,4-D and 4.5 μM kinetin-supplemented medium contained a very low amount of azadirachtin. These results clearly showed the importance of growth regulators and additives on azadirachtin production. For the present statistical optimization study, five important media components—BAP, IAA, CH, MS major salts, and sucrose—were selected that promoted the proliferation of organogenic (redifferentiated) callus from zygotic embryo cultures. Prakash and Srivastava (2005) have also emphasized the importance of carbon source and MS major nutrients on azadirachtin production. However, factors like plant growth regulators and other media addenda were never taken into consideration. The objective of the investigation was to acquire the most effective medium constituents for enhanced azadirachtin production from redifferentiated zygotic embryo cultures. This cell line produces 2.33 mg/g DW of azadirachtin in non-optimized medium, but can now produce 4.97 mg/g DW as a result of this study.

Materials and Methods

Culture establishment. Immature fruits were collected in June in three consecutive years, from 2008 to 2010, from a 35-y-old neem plant growing near the campus of the Indian Institute of Technology in Guwahati, India. Fruits were washed with 1% (*v*/*v*) antiseptic savlon (Johnson & Johnson, Solan, India) solution (Chaturvedi *et al*. 2004) for 10 min followed by three to four rinses with sterile distilled water (SDW). After rinsing with 90% ethanol for 30 s, fruits were surface-sterilized using 0.1% (*w*/*v*) mercuric chloride solution for 10 min. The disinfected fruits were washed with SDW three times and dissected under a binocular microscope (Nikon SMZ-1, Tokyo, Japan) to excise the embryos. The early dicotyledonary stage of embryo was used as an explant and cultured on MS medium (Murashige and Skoog 1962) containing 3% sucrose, 0.8% agar (Hi-Media, Mumbai, India), and 9 μM BAP, 5 μM IAA, and 500 mg/l CH. The pH of the medium

was adjusted to 5.8 before autoclaving at 1.06 kg cm^{-2} and 121°C for 15 min. Four explants were cultured in 55× 15-mm pre-sterilized, disposable Petri dishes containing 10 ml MS medium. Cultures were maintained in diffuse light (20–40 μE m^{-2} s^{-1}) with a 16-h photoperiod at 25± 2°C. The Petri dishes were sealed with Parafilm (American National Can, Greenwich, CT). Callus cultures were maintained by regular subculturing at 6-wk intervals.

For optimization of azadirachtin production, 150×25-mm rimless glass tubes (Borosil, Mumbai, India) containing 20 ml medium were inoculated with 0.5 g fresh cells. For each treatment, five replicates were evaluated. Although a single subculture cycle was sufficient to remove the carryover effects of the previous medium, cells were harvested for azadirachtin estimation after the second passage onward, at 6-wk intervals.

Plackett-Burman design. The Plackett–Burman design was used to evaluate the relative importance of various nutrients on azadirachtin production. The Plackett–Burman experimental design is based on the first-order polynomial model:

$$Y = \beta_o + \Sigma\beta_i X_i$$

where Y is the response, β_o is the model intercept and β_i is the linear coefficient, and X_i is the level of independent variable. This model does not describe interaction among factors and is used to screen and evaluate the important factors that influence the response. In the present work, five assigned variables were screened with 12 treatment combinations (Table 1). Each independent variable was tested at two levels, a high (+1) level and a low (−1) level. The high level of each variable was set far enough from the low level to identify which ingredient of the medium has a significant influence on azadirachtin production. From regression analysis, the variables which were significant at the 95% level ($p<0.05$) were considered to have a greater impact on azadirachtin production. These variables were further optimized by a CCD. The experimental design was developed using Minitab 15.5 statistical software package (Statsoft, Tulsa, OK).

Response surface methodology. A CCD was employed for determining the optimum concentration level of three significant factors screened in the Plackett–Burman design. The experimental data to correlate the relationship between the response value (azadirachtin content) and the variables were explained by the following second-order polynomial model:

$$Y = \beta_o + \Sigma\beta_i X_{i+} \Sigma\beta_{ii} X_{i+}^2 \Sigma\Sigma\beta_{ij} X_i X_j$$

where Y is the predicted response, β_o is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient, and X_i and X_j are the coded independent variables or factors.

The experimental design protocol for RSM was developed using Minitab 15.5 statistical software package. The analysis of variance (ANOVA) table was generated, and the effect and regression coefficients of individual linear, quadratic, and interaction terms were determined. The significance of all the terms in the polynomial was judged statistically by computing the F value at a probability (p) of 0.05. The regression coefficients were used to make statistical calculations to generate response surface curves from the regression models.

To test the model accuracy, R^2, adjusted R^2 (R_{adj}^2) and predicted R^2 (R_{pred}^2) were estimated. For normality assumption, the Kolmogorov–Smirnov normality test was performed and outliers were checked by studentized residual values. The second-order polynomial equation was maximized using

Table 1. Plackett–Burman design showing five variables with real values along with the observed results of azadirachtin content

Run order	Sucrose (%) Low—1 High—5	MS major salts Low—0.5 High—2	BAP (μM) Low—5 High—13	IAA (μM) Low—2.5 High—7.5	CH (mg/l) Low—250 High—1000	Azadirachtin amount (mg/g DW)
1	5.00	2.00	5.00	2.50	250.00	0.25
2	5.00	2.00	13.00	7.50	250.00	3.02
3	1.00	0.50	5.00	2.50	250.00	0.13
4	1.00	2.00	5.00	7.50	1000.00	1.53
5	1.00	2.00	13.00	2.50	1000.00	0.03
6	1.00	2.00	13.00	2.50	250.00	0.04
7	5.00	0.50	13.00	7.50	250.00	3.56
8	5.00	0.50	5.00	2.50	1000.00	2.16
9	1.00	0.50	13.00	7.50	1000.00	2.08
10	5.00	2.00	5.00	7.50	1000.00	4.55
11	5.00	0.50	13.00	2.50	1000.00	3.38
12	1.00	0.50	5.00	7.50	250.00	0.65

the Minitab response optimizer under a global solution of desirability equal to 1 to obtain the optimal levels of the independent variables and the predicted maximum azadirachtin production. The accuracy of the values was verified by comparing the predicted values obtained with the mathematical model and the measured values obtained after the experiments under the same conditions.

Preparation of standard solution. An azadirachtin standard was procured from Sigma-Aldrich (St. Louis, MO). The stock solution of 1,000 μg/ml was prepared by dissolving 0.5 mg of the compound in 0.5 ml of HPLC grade methanol. The solution was then stored at −20°C. The stock solution was serially diluted with HPLC grade methanol to make samples with concentrations of 250, 125, 62.5, 31.3, 15.6, and 7.8 μg/ml. Each concentration of standard was filtered through a 0.20-μm membrane filter before HPLC analysis.

Preparation of sample solution. To prepare samples, fresh calli were harvested from various media and dried separately in an oven at 30±2°C until a constant weight was achieved. The dried cell biomass was then dipped in methanol overnight and sonicated for 45 min at 35% amplitude with 5-s pulse on and off. Samples were centrifuged in a high-speed refrigerated centrifuge (Sigma 4K15C, Osterode Am Harz, Göttingen, Germany) at 5,000 rpm for 10 min. The supernatant was pooled and water was added in the ratio of 40:60 (40% water and 60% methanol). After the addition of water, the solution was partitioned against 100 ml dichloromethane (DCM) in separating funnels. After being shaken thoroughly, separating funnels were kept aside for 10 min to separate two immiscible solvents (methanol + water and DCM). Later, the upper water–methanol layer was discarded and the DCM layer collected and evaporated to dryness at 40°C in a rotatory evaporator (Buchi Rotavapor R-200, Tokyo, Japan). The DCM fraction residue was redissolved in HPLC grade methanol, filtered through a 0.20-μm membrane filter prior to analysis, and 20 μl of the clean solution was analyzed via HPLC.

Chromatographic conditions. High-performance liquid chromatography was conducted using a Varian Prostar HPLC system (Varian, Palo Alto, CA) that consisted of a UV–visible spectrophotometer detector, a prostar binary pump, a 20-μl injection loop, and a Hypersil BDS RP-C18 column (Thermo, Waltham, MA) of dimensions 250× 4.6 mm. The mobile phase used was 90% methanol and 10% water at a flow rate of 0.5 ml/min. UV detection was carried out at 210 nm with attenuation of 0.1 absorbance units at full scales. The chromatographic peaks of the analytes were confirmed by comparing their retention time with those of the azadirachtin standards.

A calibration curve was generated by plotting the peak area (y) against concentration in micrograms per milliliter of the standard solutions (x). The standard equation obtained from the curve as $y=1.0194x+54.22$ was used for the quantification of azadirachtin in the unknown samples. Azadirachtin content was reported as milligrams per gram DW of sample. The correlation coefficients ($R^2=0.9638$) were also generated in Excel (Microsoft, Redmond, WA) by fitting the linear trend lines to the standard curves.

Analytical grade dichloromethane and methanol, and HPLC grade methanol used for analysis were purchased from Merck, Mumbai, India. Purified water used for HPLC analysis was obtained from Milli Q system (Millipore, Billerica, MA).

Results and Discussion

Evaluation of factors affecting azadirachtin production. The first optimization step was a 12-run Plackett–Burman design to identify the significant factors affecting azadirachtin production in redifferentiated zygotic embryo callus. A wide variation in azadirachtin content from 0.03 to 4.55 mg/g DW was determined in the 12 trials (Table 1). This variation reflected the significance of factors. The analysis of regression coefficients and the t value of the five medium components (Table 2) demonstrated that sucrose (X_1), MS major salts (X_2), and BAP (X_3) had significant effects on azadirachtin production. CH (X_4) and IAA (X_5) were found to be insignificant with positive coefficients. Neglecting the variables which were insignificant, the first-order model equation for azadirachtin production can be written as:

$$Y = 3.20 + 1.24X_1 - 0.73X_2 + 0.85X_3$$

With the help of relative ranking, MS major salts, sucrose, and BAP were selected for further optimization, which had the most significant effects on azadirachtin production.

Optimization of culture conditions by RSM. The three components—MS major salts, sucrose, and BAP—were optimized using response surface methodology. The re-

Table 2. Statistical analysis of Plackett–Burman design showing coefficient values and t and p values for each variable for azadirachtin production

Term	Coefficient	t	p
Constant	3.20	15.97	0.000
Sucrose	1.24	6.19	0.001
Major	−0.73	−3.65	0.011
BAP	0.85	4.25	0.005
IAA	0.09	0.43	0.680
CH	0.14	0.70	0.510

sponse surface design methods mainly comprise the CCD, the Box–Behnken design (BBD), and the D-optimal design. Of these, the CCD and the BBD are the most commonly used response surface design methods. The CCD is often suggested for sequential experimentation and is appropriate for assessing the first- and second-order terms. BBD, on the other hand, can be used for performing non-sequential experiments because it does not employ an embedded factorial design. The CCD has axial points outside the design periphery. These axial points display a significant role toward design precision; still, they are not chosen in many cases in which these conditions are away from the safe operating limits. Though in BBD all factors are set within the experimental margin, this method has a lower accuracy than CCD (Myers and Montgomery 2002). The CCD and the BBD have five levels and three levels for each factor, respectively. D-Optimal design is generally used when CCD or BBD cannot be used because of limited resources or factor setting constraints. In the present study, CCD was used to optimize the levels of significant variables, which will be useful for the mathematical model. The various combinations of the three screened factors and the corresponding measured and predicted azadirachtin contents (Table 3) show that the amounts of other variables were the same as those in the basal media.

Data were analyzed using Minitab 15.5 statistical software package and mathematical expression of relationship to the azadirachtin production with variables, which is shown below:

$$Y = 4.85 + 0.69X_1 + 0.30X_2 + 0.37X_3 - 0.06X_1X_2 + 0.00X_1X_3 + 0.14X_2X_3 - 0.65{X_1}^2 - 0.75{X_2}^2 - 0.56{X_3}^2$$

To test the fit of the model equation, the regression-based determination R^2 coefficient was evaluated. The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R^2 value is always between 0 and 1 (Haider and Pakshirajan 2007; Liu and Wang 2007). The model presented a high determination coefficient (R^2=0.9582) explaining 95.82% of the variability in the azadirachtin production. The adjusted determination coefficient (${R_{adj}}^2$) and predicted determination coefficient (${R_{pred}}^2$) were 0.9207 and 0.6776, respectively. The ${R_{adj}}^2$ corrects the R^2 value for the sample size and for the number of terms in the model. The normality test was also carried out for judging the model adequacy, which

Table 3. CCD experimental design matrix of three variables in real units and amount of azadirachtin production

Run order	Sucrose	MS major salts	BAP	Azadirachtin amount (mg/g DW)		Studentized residuals
				Measured	Predicted	
1	2.25	1.50	5.00	1.89	2.05	−1.00
2	4.50	1.00	9.00	4.86	4.86	1.23
3	4.50	1.00	9.00	4.90	4.86	1.28
4	4.50	1.00	9.00	4.89	4.86	1.27
5	6.75	0.50	13.00	3.86	3.57	−0.66
6	2.25	0.50	5.00	1.98	1.6	−0.70
7	4.50	1.00	9.00	4.88	4.86	1.26
8	6.75	0.50	5.00	3.43	3.12	−0.44
9	4.50	1.00	15.72	4.10	3.88	−0.23
10	4.50	1.84	9.00	3.48	3.23	−0.67
11	2.25	0.50	13.00	1.93	2.07	−1.18
12	4.50	1.00	9.00	4.82	4.86	1.18
13	4.50	1.00	9.00	4.82	4.86	1.18
14	8.28	1.00	9.00	3.86	4.17	−0.88
15	6.75	1.50	13.00	4.08	4.32	−0.82
16	2.25	1.50	13.00	2.87	3.06	−0.90
17	0.72	1.00	9.00	1.98	1.85	−0.80
18	4.50	1.00	2.27	2.24	2.65	−0.90
19	4.50	0.16	9.00	1.80	2.23	−1.28
20	6.75	1.50	5.00	3.58	3.31	−0.66

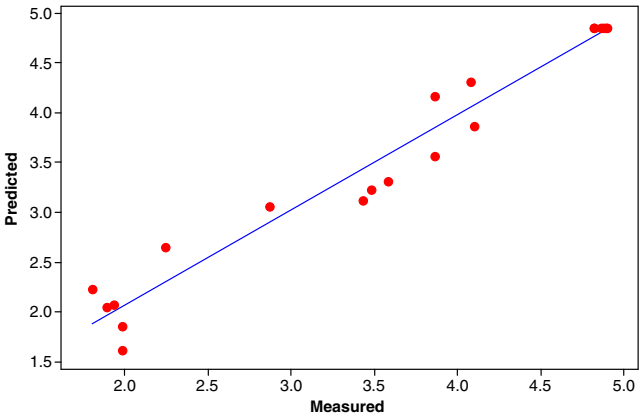

Figure 1. Linear correlation plot between measured *vs*. predicted azadirachtin content (milligrams per gram DW).

showed a p value >0.15, thus confirming the normality assumption. To check the outliers, the studentized residual values were calculated. All the values lie within the range of −2 and +2, thereby validating the model (Table 3). According to Anderson and Whitcomb (2005), studentized residual values higher than −3.5 and +3.5 are considered as outliers.

The correlation plot was made between the measured values of azadirachtin content and the predicted (modeled) values determined by the model (Fig. 1). For each variable, model coefficients were estimated by regression analysis (Table 4). The significance of each coefficient was determined by t values and p values. The larger t value and the smaller p value indicate the high significance of the corresponding coefficient (Karthikeyan *et al*. 1996; Tanyildizi *et al*. 2005). A value of $p<0.05$ implies that the model is significant. The results revealed that sucrose concentration, MS major salts, and BAP had a significant effect on azadirachtin production. Positive coefficients of sucrose, MS major salts, and BAP variables indicated a linear effect for the increase in azadirachtin production. Among the interactions, sucrose × BAP ($p<1.000$) and

Table 4. Regression analysis of CCD for azadirachtin production

Term	Coefficient	t	p
Constant	4.85	35.69	0.000
Sucrose	0.69	7.66	0.000
MS major salts	0.30	3.28	0.008
BAP	0.37	4.05	0.002
Sucrose × sucrose	−0.65	−7.43	0.000
MS major salts × MS major salts	−0.75	−8.55	0.000
BAP × BAP	−0.56	−6.42	0.000
Sucrose × MS major salts	−0.06	−0.51	0.622
Sucrose × BAP	0.00	−0.00	1.000
MS major salts × BAP	0.14	1.17	0.271

Table 5. Analysis of variance of CCD for optimization of azadirachtin production

Source	*DF*	SS	MS	*F*	*p*
Regression	9	25.53	2.84	25.50	0.000
Linear	3	9.54	3.18	28.61	0.000
Square	3	15.80	5.27	47.36	0.000
Interaction	3	0.18	0.06	0.54	0.666
Pure error	5	0.01	0.00		
Total	19	26.65			

R^2 =95.82%; R_{adj}^2 =92.07%; R_{pred}^2 =67.76%

SS sum of squares, *DF* degrees of freedom, *MS* mean square

major salts × BAP ($p<0.271$) had positive coefficients; sucrose × major salts ($p<0.622$) had negative coefficients. To validate the regression coefficient, analysis of variance for azadirachtin production was performed (Table 5).

The graphical depiction provides a method to visualize the relationship between the response and experimental levels of each variable and the type of interactions between test variables to deduce the optimum conditions. One such response surface representing azadirachtin production, in the present study, was a function of the concentrations of the two medium components with a third nutrient being at an optimum level (Fig. 2). A steep slope or curvature shows that azadirachtin production is sensitive to that factor.

The model predicted a maximum azadirachtin content of 5.13 mg/g DW by solving the regression equation and also by analyzing the response surface plot by Minitab software. The optimum levels of the significant variables were: sucrose, 5.68%; BAP, 10.42 μM; and full MS major salts. To validate the predicted model, three experiments were conducted using this optimum medium composition. Azadirachtin content of

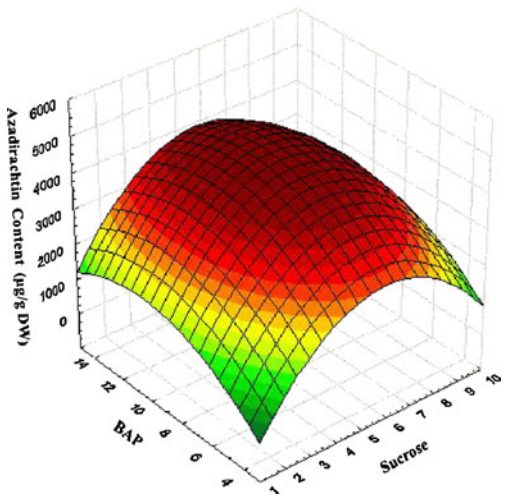

Figure 2. Response surface curves of azadirachtin production showing interaction between sucrose and BAP, fixed level: MS major salts at middle level.

4.97 mg/g DW was observed at this medium composition, which agreed well with the predicted value (5.13 mg/g DW). As a result, the developed model was considered to be accurate and reliable for predicting the production of azadirachtin from *in vitro* cell lines of neem.

Conclusion

Azadirachtin production from redifferentiated zygotic embryo cultures was optimized by RSM. Among the variables studied, sucrose, MS major salts, and BAP were found to significantly affect azadirachtin production. Under optimal medium compositions (full MS major salts, 5.68% sucrose, and 10.42 μM BAP), the experimental value of 4.97 mg/g DW closely matched the predicted value of 5.13 mg/g DW. The experimental value was 2.16 times higher than the control medium in which 2.33 mg/g DW of azadirachtin was produced.

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