

Optimization of shikonin production in *Onosma dichroantha* callus using response surface methodology

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Abstract The roots of *Onosma dichroantha*, contain substantial amounts of shikonin/alkannin derivatives, which are considered as edible natural colors with distinguished anti-inflammatory, antibacterial, antiviral, as well as anti-tumor activities. In this study, a response surface methodology coupled with numerical optimization modeling techniques were implemented to analyze and optimize the individual as well as interactive effects of two important plant growth regulators (indole acetic acid, IAA and benzylaminopurine, BAP) on the relative growth rate (RGR) and shikonin production of *O. dichroantha* callus grown on three growth media. Callus growth was best on B5 medium, and shikonin production was best on M9 medium, while White medium was best for synchronized callus growth and shikonin production. Upon statistical optimization, the maximum RGR and shikonin production of callus grown on B5 medium were predicted in 0.93 mg L⁻¹ BAP and 0.16 mg L⁻¹ IAA, on M9 medium in 0.55 mg L⁻¹ BAP and 0.16 mg L⁻¹ IAA, and on White medium in 1.23 mg L⁻¹ BAP and 0.11 mg L⁻¹ IAA. These data can be employed in development of large scale production of shikonin derivatives using *O. dichroantha*.

Keywords Benzylaminopurine · Boraginaceae · Indole acetic acid · *Onosma dichroantha* · Response surface design · Shikonin

Introduction

Onosma dichroantha Boiss. belongs to the Boraginaceae family which are mainly distributed in dry, cliffy and sunny habitats of Eurasia, Mediterranean and western North America; with a maximum diversity in Iran (Willis 1973). *O. dichroantha* and other *congeners* are considered as potential sources of important pharmaceutical agents, such as shikonin/alkannin, with strong antioxidant, antibacterial, antiviral and anticancer activities (Chiu and Yang 2007; Kretschmer et al. 2012; Liu et al. 2012; Rinner et al. 2010). Root extracts of *O. dichroantha* have long been used in Iran as a remedy for burn-wound healing, however, the market value of the plant is rather limited because of its low natural germination and propagation rates (our unpublished data). The distribution of *O. dichroantha* in Mazandaran, Iran is decreasing due to the extensive harvest of its roots and degradation of its natural habitats, by increased agricultural activities (Hamzeh'ee et al. 2010).

Micropropagation is considered as a useful method for in vitro cultivation, offering the development of thousands of plants with desired specifications, which might be further explored for metabolic engineering through molecular biotechnology techniques. This method has been successfully developed for some boraginaceous plants for in vitro production of shikonin/alkannin derivatives (Fujita et al. 1981; Liu et al. 2012; Wu et al. 2009), however, to our knowledge, in vitro cultivation of *O. dichroantha* for shikonin production has not yet been studied.

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Recently, response surface methodology (RSM) has been successfully employed to optimize callus production and plant regeneration of various species (Gómez-Montes et al. 2015; Gutiérrez Miceli et al. 2007; Hand and Reed 2014; Niedz et al. 2014; Reed et al. 2013). In this method, a collection of statistical measures, including detection of important independent variables and their effectiveness, selection of a proper experimental design, prediction and verification of various polynomial models, generation of contour plots and 3D response surfaces, and finally determination of optimum values for the factors, are employed to produce desired responses (Chakraborty et al. 2010; George et al. 2000; Hanchinal et al. 2008; Montgomery 1997).

According to previous experiments, the induction rate, growth and color of callus in the Boraginaceae family highly depend on the plant species, type of explant used, composition of the growth media, and the presence of various plant growth regulators (Koca et al. 2012; Malik et al. 2016). In addition, optimum conditions for callus establishment and growth might be quite different from those for shikonin derivatives production (Wu et al. 2009). Various growth media, including B5 (Gamborg et al. 1968), M9 (Fujita et al. 1981) and White (White 1934), have been proposed for callus propagation and/or shikonin production for boraginaceous plants. The White growth medium was one of the first rich growth media containing various inorganic and organic nutrients, including yeast extract, which was introduced for long time in vitro culture of tomato root tips (White 1934, 1954). On the other hand, Gamborg B5 is a simple growth medium consisting of mineral salts, sucrose, vitamins and 2,4-dichlorophenoxyacetic acid (2,4-D), without any complex compounds like yeast extract or coconut milk, initially used for soybean tissue culture (Gamborg et al. 1968). The M9 is a derivative of White medium, optimized for shikonin production of a Boraginaceous plant *Lithospermum erythrorhizon*, in which the concentrations of copper, nitrate and sulfate are increased 30, 3, and 2 times, respectively, while ammonia, manganese, iodate, glycine, thiamine hydrochloride, pyridoxine hydrochloride and nicotinic acid were removed, as compared to White medium (Fujita et al. 1981). Changing the growth medium, to increase shikonin production, is tedious and might compromise the growth rate of the callus itself. Besides, the best concentration of various plant growth regulators for callus induction and proliferation in those growth media has not yet been carefully optimized. The present study was conducted to determine the optimum levels of two important plant growth regulators, auxin (indole acetic acid, IAA) and cytokinin (benzylaminopurine, BAP) in the B5, M9 and White growth media, in order to develop a reliable protocol for in vitro propagation and shikonin production in *O. dichroantha*.

Materials and methods

Seed disinfection and cultivation

Onosma dichroantha Boiss seeds, collected from Galoogah Mountains (Mazandaran, Iran, N36°41'55"; E53°50'24"), were washed with water containing 1 % tween 20 for 15 min, rinsed three times with sterile distilled water and surface sterilized in 70 % (v/v) ethanol and 20 % (v/v) commercial bleach for 5 and 10 min, respectively, followed by three washes with sterile distilled water. Subsequently, the seed coats were carefully removed and the embryos were again sterilized in 70 % ethanol for 30 s and 20 % bleach for 2 min and washed five times by sterile distilled water. Embryos were sown in ordinary glass bottles (6 × 10 cm) containing 40 ml of sterilized Murashige and Skoog (MS) mineral medium (Murashige and Skoog 1962), supplemented with 3 % sucrose (w/v), and solidified with 0.7 % agar. Bottles were incubated in a growth chamber under cool white fluorescent light (45 μmol quanta m⁻² s⁻¹) and 16 light/8 dark photoperiod at 22–25 °C for about 2 months for embryo germination and seedling growth.

Callus induction and growth

For callus induction, leaf and root explants were cut in about 10 mm sections, aseptically and placed on Petri dishes containing MS medium (Murashige and Skoog 1962) with 3 % sucrose (w/v), 0.7 % agar, 0.5 mg L⁻¹ of each of nicotinic acid, pyridoxine-HCl, and thiamin-HCl, together with 100 mg L⁻¹ of myo-inositol, without any plant growth regulator. The Petri dishes were incubated in a growth chamber in the dark at 22–25 °C for 60 days. Established healthy callus were isolated from the explants and propagated on Petri dishes containing 30 ml of either B5 (Gamborg et al. 1968), M9 (Fujita et al. 1981) or White (White 1934) growth media with five concentrations of IAA and BAP, as indicated in Table 1 and were incubated in a growth chamber in the dark at 22–25 °C for 30 days, after which the fresh weight of the callus were determined and used in the following equation to calculate the relative growth rate (RGR) as the response.

$$RGR = \frac{W_2 - W_1}{W_1} \times \frac{1}{T_2 - T_1} \quad (1)$$

Where, W shows the fresh weight (g); and T is the time (day).

Shikonin measurement

Shikonin and its derivatives were initially measured spectrophotometrically (Shimadzu UV-1800, Japan) as

Table 1 Uncoded values of the independent variables (BAP and IAA) in response surface design matrix and the observed and model predicted results for callus relative growth rates (RGR) in three different growth media (B5, M9 and White)

Run order	BAP, X_1 (mg L^{-1})	IAA, X_2 (mg L^{-1})	RGR B5		RGR M9		RGR white	
			Actual	Predicted	Actual	Predicted	Actual	Predicted
1	1.71	0.17	0.065	0.066	0.021	0.020	0.033	0.034
2	1.00	0.20	0.070	0.067	0.016	0.017	0.031	0.035
3	0.00	0.10	0.029	0.030	0.013	0.012	0.015	0.014
4	0.00	0.10	0.028	0.030	0.010	0.012	0.013	0.014
5	1.00	0.00	0.055	0.056	0.018	0.019	0.029	0.027
6	0.29	0.03	0.047	0.041	0.015	0.015	0.019	0.018
7	1.00	0.00	0.052	0.056	0.022	0.019	0.023	0.027
8	1.00	0.20	0.068	0.067	0.014	0.017	0.038	0.035
9	1.71	0.03	0.055	0.055	0.018	0.021	0.027	0.028
10	0.29	0.17	0.049	0.045	0.016	0.014	0.023	0.024
11	1.71	0.17	0.062	0.066	0.019	0.020	0.034	0.034
12	1.00	0.10	0.082	0.083	0.026	0.022	0.034	0.036
13	2.00	0.10	0.054	0.054	0.022	0.021	0.027	0.028
14	0.29	0.17	0.047	0.045	0.014	0.014	0.025	0.024
15	2.00	0.10	0.050	0.054	0.020	0.021	0.030	0.028
16	0.29	0.03	0.041	0.041	0.016	0.015	0.016	0.018
17	2.00	0.10	0.057	0.054	0.023	0.021	0.026	0.028
18	1.00	0.10	0.086	0.083	0.022	0.022	0.036	0.036
19	0.29	0.03	0.042	0.041	0.015	0.015	0.020	0.018
20	1.00	0.20	0.064	0.067	0.018	0.017	0.034	0.035
21	0.29	0.17	0.043	0.045	0.013	0.014	0.022	0.024
22	1.71	0.03	0.058	0.055	0.020	0.021	0.031	0.028
23	1.00	0.10	0.081	0.083	0.020	0.022	0.035	0.036
24	1.71	0.17	0.069	0.066	0.022	0.020	0.038	0.034
25	1.00	0.00	0.055	0.056	0.017	0.019	0.028	0.027
26	1.00	0.10	0.088	0.083	0.022	0.022	0.040	0.036
27	1.00	0.10	0.079	0.083	0.020	0.022	0.033	0.036
28	1.71	0.03	0.054	0.055	0.023	0.021	0.025	0.028
29	0.00	0.10	0.026	0.030	0.012	0.012	0.016	0.014

previously described (Gupta et al. 2014) and confirmed by HPLC basically as described (Sagrati et al. 2008), using a Hitachi (D-7000, Series 0127, Japan) HPLC–DAD instrument, equipped with a binary solvent pump (L 7100) and a DAD (diode array detector).

Experimental design and statistical analysis

Among various classes of RSM designs, the central composite design (CCD) is very popular mainly because it can be run sequentially in an efficient and flexible manner (Montgomery 1997). Design-Expert 7.0 (Stat-Ease, Minneapolis, MN) software was used for designing the experiments and analyzing the data in the CCD mode which was used to statistically develop the models and to evaluate the direct, pairwise, and curvilinear effects of

the plant growth regulator variables (BAP and IAA) on callus RGR and shikonin production in each growth medium. Based on preliminary experiments BAP ($0\text{--}2\text{ mg L}^{-1}$) and IAA ($0\text{--}0.2\text{ mg L}^{-1}$) were used at five levels in a 2^2 full factorial CCD-RSM, consisting of four factorial cube points and four axial points (each in triplicate) as well as five replicates at the center point of the cube (for estimation of pure error) which aggregated 29 experimental runs. Experiments were run in randomized order to minimize the effects of unexplained extraneous factors.

Multiple regression analyses and Pareto analysis of variance (ANOVA) were used to analyze the experimental data and ANOVA tables were generated. The adequacy of the developed models were tested by performing coefficient of determination (R^2), adjusted coefficient of

determination (R_{adj}^2) and predicted coefficient of determination (R_{pre}^2) analyses. After fitting the models, the generated data were employed for plotting 3D response surfaces and optimization contour plots.

Results

Various concentrations of BAP and IAA have been used for callus proliferation (Fig. 1) as well as shikonin production of *O. dichroantha* in the B5, M9 and White growth media using the CCD-RSM methodology. The uncoded different combinations of experimental conditions with the respective experimental responses (callus RGR and shikonin production) alongside the predicted values obtained

from the final mathematical models are presented in Tables 1 and 2.

Central composite analysis

To obtain the best preliminary regression models for each growth medium, the experimental data were analyzed and initially fitted to the following full quadratic model.

$$Y = \beta_1 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \varepsilon \quad (2)$$

Where, Y is the response; $\beta_{1,2,\dots,k}$ are coefficients of variables; and $X_{1,2,\dots,i}$ are the variables.

The regression analysis of this basic model for each medium revealed the significance of the effects of each

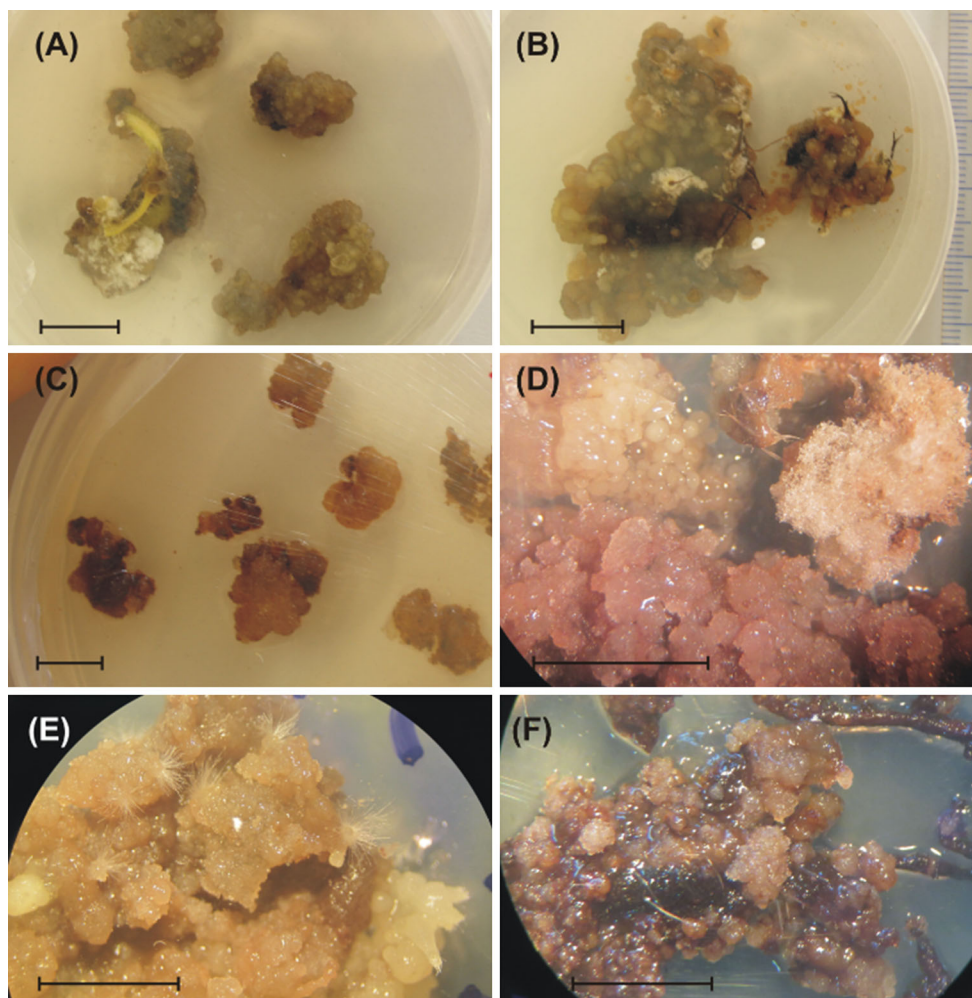


Fig. 1 Callus induction and proliferation of *Onosma dichroantha* on various media. Callus initiation on MS medium, 50 days after incubation in the dark (a). Callus proliferation on B5 medium, 30 days after incubation in the dark (b). Callus proliferation on M9

medium 10 days (c) and 30 days (d) after incubation in the dark (notice the pink color which is a sign of shikonin production). Callus proliferation on White medium 10 days (e) and 30 days (f) after incubation in the dark. Scale bars indicate 1 cm

Table 2 Uncoded values of the independent variables (BAP and IAA) in response surface design matrix and the observed and model predicted results for Shikonin production by *O. dichroantha* in three different growth media (B5, M9 and White)

Run order	BAP, X_1 (mg L ⁻¹)	IAA, X_2 (mg L ⁻¹)	Shikonin B5		Shikonin M9		Shikonin white	
			Actual	Predicted	Actual	Predicted	Actual	Predicted
1	1.71	0.17	28.29	33.17	44.36	38.15	39.51	44.50
2	1.00	0.20	33.74	38.36	61.62	66.36	36.92	37.11
3	0.00	0.10	29.78	33.93	85.73	84.02	28.68	32.57
4	0.00	0.10	32.64	33.93	83.17	84.02	29.43	32.57
5	1.00	0.00	25.09	22.21	46.74	48.52	38.13	44.62
6	0.29	0.03	18.10	17.97	51.50	53.79	49.12	42.99
7	1.00	0.00	17.83	22.21	49.32	48.52	45.98	44.62
8	1.00	0.20	40.95	38.36	67.53	66.36	35.75	37.11
9	1.71	0.03	33.49	37.51	57.75	55.07	45.66	40.59
10	0.29	0.17	45.55	45.14	101.21	95.95	23.32	28.45
11	1.71	0.17	35.52	33.17	38.81	38.15	42.61	44.50
12	1.00	0.10	26.50	30.28	54.18	45.23	52.28	50.48
13	2.00	0.10	43.76	39.29	42.87	44.06	48.53	42.22
14	0.29	0.17	40.67	45.14	94.62	95.95	29.53	28.45
15	2.00	0.10	39.17	39.29	37.63	44.06	36.63	42.22
16	0.29	0.03	24.85	17.97	55.35	53.79	45.93	42.99
17	2.00	0.10	37.39	39.29	47.72	44.06	45.12	42.22
18	1.00	0.10	27.69	30.28	47.34	45.23	52.87	50.48
19	0.29	0.03	14.92	17.97	48.46	53.79	38.64	42.99
20	1.00	0.20	42.84	38.36	68.44	66.36	40.34	37.11
21	0.29	0.17	48.28	45.14	89.36	95.95	34.29	28.45
22	1.71	0.03	37.58	37.51	56.91	55.07	33.75	40.59
23	1.00	0.10	35.73	30.28	42.57	45.23	49.57	50.48
24	1.71	0.17	31.47	33.17	35.52	38.15	47.46	44.50
25	1.00	0.00	19.25	22.21	52.84	48.52	47.27	44.62
26	1.00	0.10	31.31	30.28	36.50	45.23	46.32	50.48
27	1.00	0.10	32.44	30.28	45.55	45.23	51.38	50.48
28	1.71	0.03	42.13	37.51	51.37	55.07	41.39	40.59
29	0.00	0.10	37.18	33.93	88.95	84.02	35.17	32.57

plant growth regulator, and also their linear and quadratic interactions on the responses.

Model fitting

To further adjust and fine tune a quadratic model that better explains the relationship between the independent (BAP, X_1 and IAA, X_2) and the dependent variables (callus RGR and shikonin production), the CCD-RSM fitted models were carefully analyzed by a stepwise regression analysis ($\alpha = 0.10$) and the final model for each medium was developed. According to this analysis, Eq. 3, using uncoded units and $R^2 = 97.00$ and $R^2_{\text{adj}} = 96.34$, represents the best model for prediction of callus RGR and Eq. 4, using

uncoded units with $R^2 = 83.51$ and $R^2_{\text{adj}} = 80.77$ represents the best model for shikonin production in the B5 medium.

$$\begin{aligned} RGR_{B5} = & 0.0158 + 0.453 IAA + 0.091 BAP - 2.153 IAA^2 \\ & + 0.033 IAA BAP - 0.041 BAP^2 \end{aligned} \quad (3)$$

$$\begin{aligned} Shikonin_{B5} = & 10.10 + 5.78 BAP + 238.31 IAA \\ & - 157.58 IAA BAP + 6.33 BAP^2 \end{aligned} \quad (4)$$

For the M9 medium, the interaction effects of IAA \times BAP were not significant for callus RGR and therefore removed from the equation, leading to the final model using data in uncoded units (Eq. 5) with $R^2 = 79.37$

and $R_{\text{adj}}^2 = 75.94$.

$$\begin{aligned} \text{RGR M9} = & 0.009 + 0.075 \text{ IAA} + 0.015 \text{ BAP} - 0.420 \text{ IAA}^2 \\ & - 0.005 \text{ BAP}^2 \end{aligned} \quad (5)$$

Equation 6 represents the best full quadratic model using uncoded units for shikonin production in the M9 medium with $R^2 = 95.17$ and $R_{\text{adj}}^2 = 94.12$.

$$\begin{aligned} \text{Shikonin M9} = & 57.78 + 140.29 \text{ IAA} - 28.07 \text{ BAP} \\ & - 295.37 \text{ IAA BAP} + 1221.45 \text{ IAA}^2 \\ & + 18.81 \text{ BAP}^2 \end{aligned} \quad (6)$$

Similar to the M9 growth medium, for callus RGR in the White growth medium, the interaction effects of IAA \times BAP was statistically negligible, thus, removed from the model, leading to the Eq. 7 as the final model with $R^2 = 90.98$ and $R_{\text{adj}}^2 = 89.47$, using uncoded units.

$$\begin{aligned} \text{RGR White} = & 0.005 + 0.014 \text{ IAA} + 0.036 \text{ BAP} \\ & - 0.498 \text{ IAA}^2 - 0.014 \text{ BAP}^2 \end{aligned} \quad (7)$$

The best model for shikonin production in the White medium is presented in Eq. 8 with $R^2 = 74.34$ and $R_{\text{adj}}^2 = 68.76$, using uncoded units.

$$\begin{aligned} \text{Shikonin White} = & 35.93 + 62.55 \text{ IAA} + 21.78 \text{ BAP} \\ & - 92.22 \text{ IAA BAP} - 961.6 \text{ IAA}^2 \\ & - 13.09 \text{ BAP}^2 \end{aligned} \quad (8)$$

Statistical analysis

The ANOVA analysis of the above indicated models (Supplementary Table 1) revealed the relationship between the effects of independent variables on the responses in each culture medium. The significance of each term was evaluated by its P value and showed that all proposed models were highly significant ($P < 0.05$) for all three media (Table 3). In each case, the F values were calculated and found to be high, indicating that the computed Fisher's variance ratios at this level were large enough to justify a high degree of adequacy of the adjusted quadratic models and also to show that the parameters selected in each model were appropriate and significant (Table 3). "Adequacy precision" measures the signal to noise ratio and a ratio greater than 4 is desirable. In our experiments, the adequacy precision for B5, M9 and White growth media for both callus RGR and shikonin production were significantly more than 4 (Table 3). The R_{adj}^2 values indicate the fraction of variation of the response explained by the model (Montgomery 1997). For all three media, the R_{adj}^2 values for callus RGR and shikonin production, which could be noticeably smaller than R^2 , are also high and suggest high

significance of the models (Table 4). In addition, high R_{pre}^2 values for callus RGR and shikonin production in B5, M9 and White, suggest an acceptable correlation between the observed and the predicted values (Table 4). In general, the R_{adj}^2 and R_{pre}^2 should be within approximately 20 % of each other to be in reasonable agreement; otherwise, there may be a problem with either the data or the model (Montgomery 2007).

To further analyze the competence of fit for the proposed models, residual plots were analyzed and confirmed normal distribution of residuals with acceptable equal variance (data not shown). In addition, the Lack of Fit probability for the above indicated models presented in Table 3 indicated that the Lack of Fit hypothesis of the models was rejected at $P < 0.05$. Altogether, these data confirmed that the proposed models might significantly predict the effects of IAA and BAP concentrations on the RGR and shikonin production of *O. dichroantha* callus cultured on the examined growth media.

Optimization of *O. dichroantha* callus growth and shikonin production on B5, M9 and White media

In the present study, for the first time, the effects of IAA and BAP on the RGR of *O. dichroantha* callus grown on the B5, M9 and White media and their ability to produce shikonin were investigated. The 3D response surface plots of the interaction between IAA and BAP on callus RGR and shikonin production, based on the above indicated models (Eqs. 3–8) are depicted in Fig. 2. These data show that for the applied ranges of IAA and BAP, the B5 growth medium produced almost four times more callus than M9 and two times more than White growth media. Regarding shikonin production, however, the M9 medium produced almost twice as much as the other two growth media.

Figure 2a shows that optimum concentrations of IAA and BAP for callus RGR in the B5 medium were observed near the middle of the applied concentrations (BAP, 1 and IAA, 0.1 mg L⁻¹). On the other hand, in this medium, higher shikonin values were detected when the concentrations of IAA and BAP were increased to their maximum levels (Fig. 2b). In the M9 medium, increasing concentrations of BAP increased the growth rate of callus (Fig. 2c) while increased concentrations of IAA resulted in positive effects on shikonin production (Fig. 2d). In the White medium, an optimum value for BAP for callus growth and shikonin production was observed, while increased IAA had a negative effect on shikonin production (Fig. 2f) and did not show significant effects on callus growth (Fig. 2f). The White medium was originally introduced for root culture of tomato plants (White 1934). Since

Table 3 Summary of the ANOVA analysis for the models indicated in Eqs. 3–8. For comprehensive ANOVA analysis refer to Supplementary Table 1

Source model	Sum of squares	df	Mean square	F value	P value	Adequacy precision
RGR B5	7.460E–003	5	1.492E–003	148.60	<0.0001*	37.171
<i>Lack of Fit</i>	4.945E–005	3	1.648E–005	1.82	0.1766 ^{ns}	
RGR M9	3.430E–004	4	8.575E–005	23.09	<0.0001*	11.929
<i>Lack of Fit</i>	1.113E–005	4	2.782E–006	0.71	0.5925 ^{ns}	
RGR White	1.429E–003	4	3.573E–004	60.50	<0.0001*	21.080
<i>Lack of Fit</i>	7.857E–006	4	1.964E–006	0.29	0.8787 ^{ns}	
Shikonin B5	1774.49	4	443.62	30.39	<0.0001*	17.131
<i>Lack of Fit</i>	24.94	4	6.24	0.38	0.8179 ^{ns}	
Shikonin M9	9489.96	5	1897.99	90.61	<0.0001*	27.762
<i>Lack of Fit</i>	41.66	3	13.89	0.63	0.6036 ^{ns}	
Shikonin White	1273.20	5	254.64	13.32	<0.0001*	11.079
<i>Lack of Fit</i>	27.85	3	9.28	0.45	0.7194 ^{ns}	

* Significant, ^{ns} not significant**Table 4** R^2 values of the models indicated in Eqs. 3–8. For details refer to Supplementary Table 1

Source model	B5		M9		White	
	RGR ^a	Shikonin	RGR	Shikonin	RGR	Shikonin
R^2	97.00	83.51	79.37	95.17	90.98	74.34
R^2_{adj}	96.34	80.77	75.94	94.12	89.47	68.76
R^2_{pre}	95.23	75.73	69.21	92.35	86.62	59.11

^a Relative growth rate

then, many researchers used this medium, or the revised version, to optimize tissue culture of various plants including Boraginaceae family members (Shekhawat and Shekhawat 2011).

Desirability analyses and model validation

In most protocols aimed at production of secondary metabolites, using boraginaceous plants, a two-stage culture system is employed in which a rich growth medium (e.g. Linsmaier–Skoog or B5) is suggested for callus induction and growth, while the M9 medium is proposed for shikonin production (Fujita et al. 1981; Gupta et al. 2014). Growth medium replacement is a time consuming and contamination prone procedure, thus, proposition of suitable concentrations of IAA and BAP for synchronized optimum callus growth and shikonin production in a single growth media is desired. The desirability function implemented in statistical modeling software such as Design-Expert is widely employed for synchronized optimization of single or multiple processes, in which the values of independent variables that provide the most desirable response (i.e. maximum RGR and shikonin production) are identified. Based on these analyses, the optimum concentrations of IAA and BAP for maximum callus RGR and shikonin production suggested by the models (Eqs. 3–8)

are presented in Fig. 3 and Table 5. According to these data, among the examined growth media, B5 was the best medium for callus proliferation and M9 was the best medium for shikonin production, while White medium was suitable for both callus proliferation and shikonin production with a the highest desirability coefficient.

To validate the models, the predicted optimized concentrations of BAP and IAA for maximum callus RGR and shikonin production (Fig. 3) were compared to their closest mean experimental values presented in Tables 1 and 2. These evaluations for all three growth media revealed close proximity of the experimental and predicted data, approving that the predicted optimal conditions for maximum callus production are correct. A desirability value more than zero implies that the response is within a desired range and a desirability value close to 1 shows that the response values are close to target values. It should be noted that these optimal values are valid within the specified range of conditions employed in the current experiments.

Discussion

In this report, we employed a response surface analysis for modelling and optimizing *O. dichroantha* callus culture. Similar methods have recently been used for optimizing

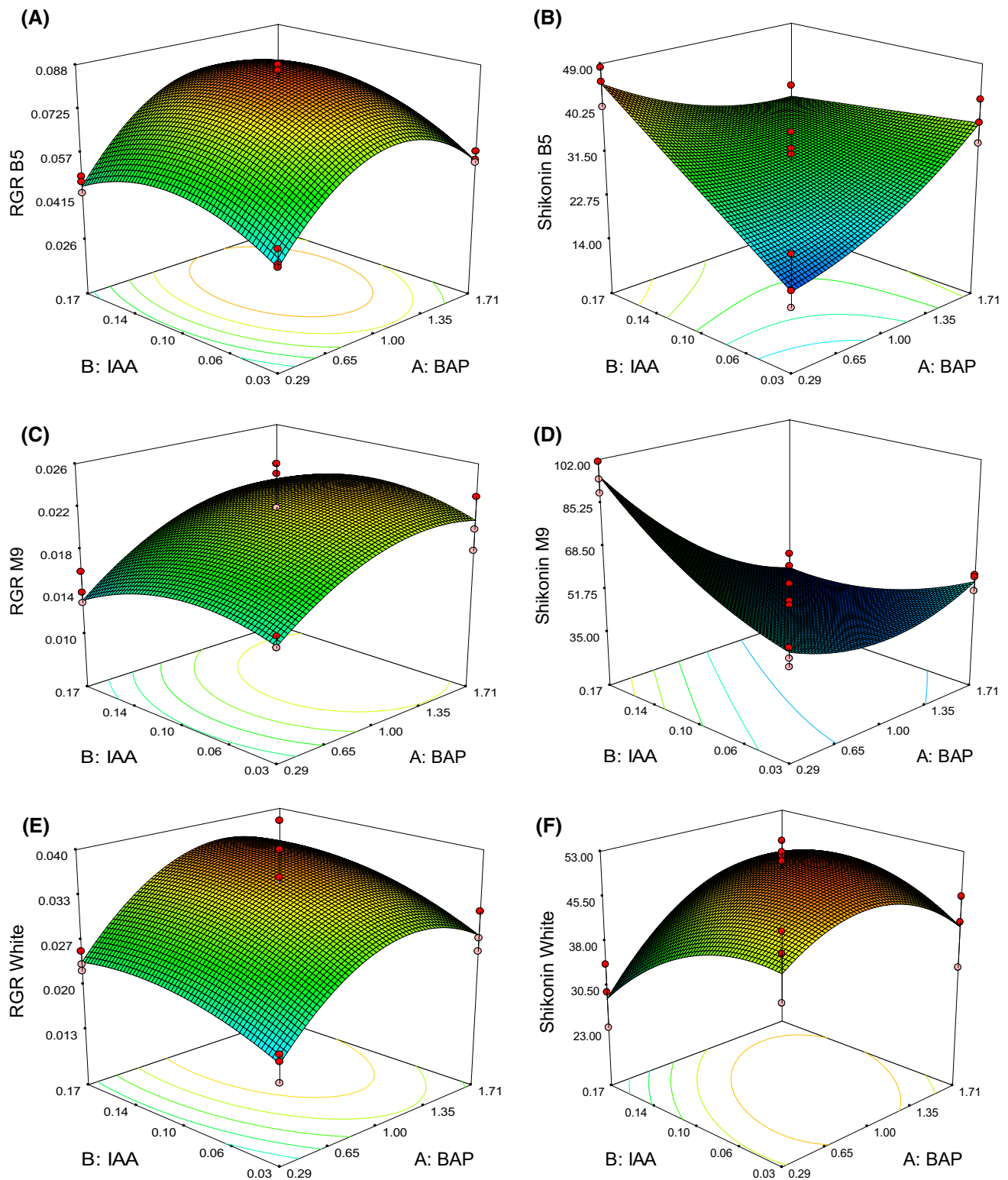


Fig. 2 Three-dimensional (3D) response surface plots showing the effects of variables (IAA and BAP) on *O. dichroantha* callus relative growth rate (RGR) and shikonin production grown on B5 (a, b), M9 (c, d) and White growth media (e, f)

callus cultures of various plant species (Gómez-Montes et al. 2015; Gutiérrez Miceli et al. 2007; Hand and Reed 2014; Niedz et al. 2014; Reed et al. 2013). This method has

two advantages over the more common factorial design: (1) RSM allows us to study the effects of independent variables between the actual experimental data points

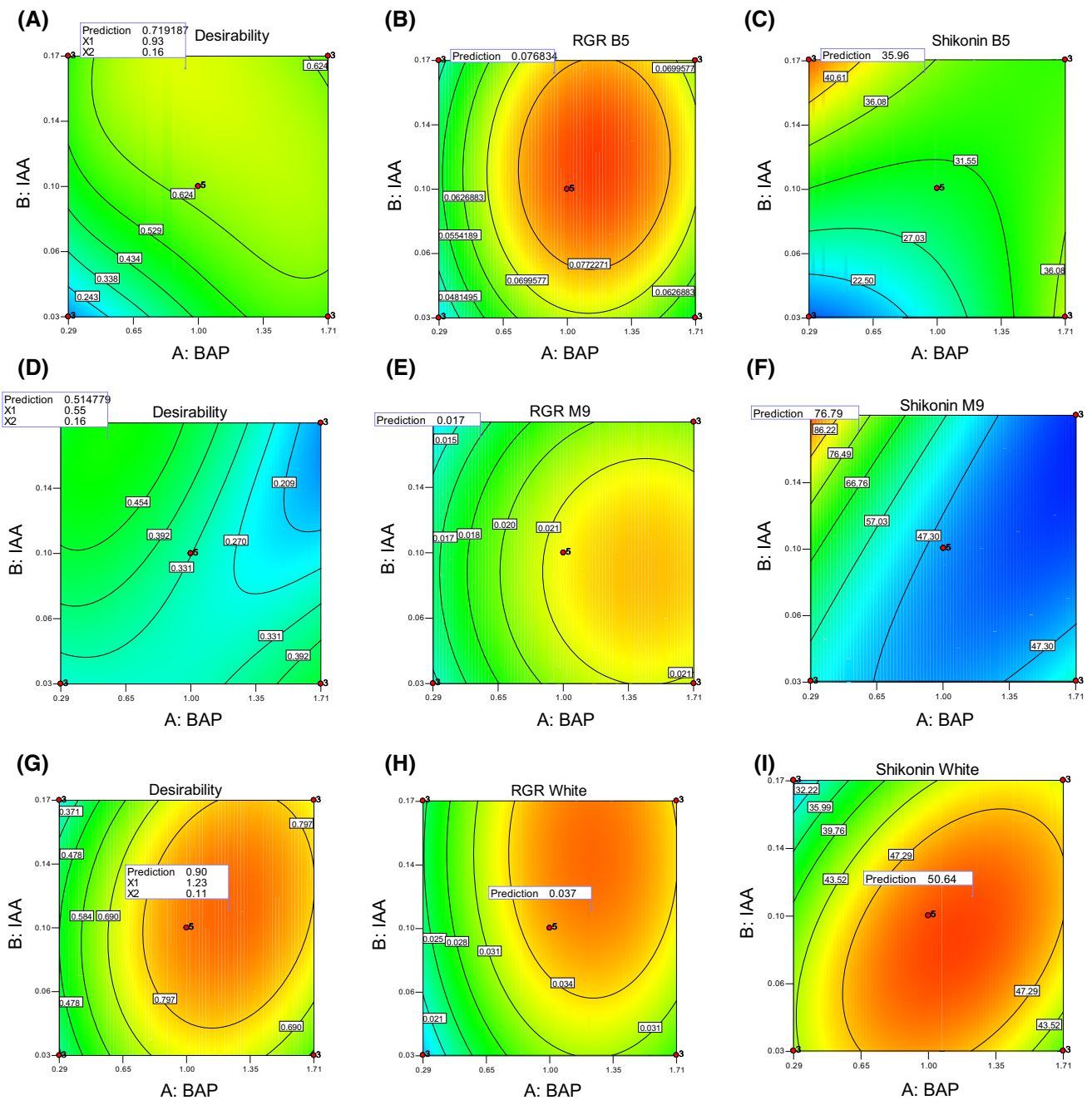


Fig. 3 Contour plots representing the desirability coefficients and predicted responses for callus RGR and shikonin production in *O. dichroantha* grown on B5 (a–c), M9 (d–f), and White media (g–i).

Blue to red contours indicate minimum to maximum desirability for the responses. The numbers close to the red small circles represent number of replicates at those data points. X1, BAP; X2, IAA

Table 5 Simultaneous optimization of callus RGR and shikonin production for *O. dichroantha*

Growth medium	BAP (mg L ⁻¹)	IAA (mg L ⁻¹)	RGR (mg L ⁻¹)	Shikonin (mg g ⁻¹ FW)	Desirability
B5	0.93	0.16	0.077	35.96	0.719
M9	0.55	0.16	0.017	76.83	0.515
White	1.23	0.11	0.037	50.64	0.903

(Montgomery 2007) and (2) it allows the researcher to easily increase the number of experimental variables to more than five, which is not practical in normal factorial designs. Our data may encourage the application of RSM in the future tissue culture optimization and in vitro production of secondary metabolites, in which an understanding of the complex interactions between plant growth regulators (PGRs) and/or growth medium nutrients is sought.

Our data showed that RGR of *O. dichroantha* callus cultured on B5 medium was twice as much cultured on White and about four times more than that cultured on M9 media. To our knowledge, this is the first report on the optimization of *O. dichroantha* tissue culture, however, differential growth rate of other Boraginaceae plants cultured on various growth media have been documented (Wu et al. 2009; Yang et al. 1999). In general, M9 medium is basically a minimal medium that puts the plant under nutritional stress conditions suitable for shikonin production (Fujita et al. 1981). In contrast, white and B5 are rich growth media optimized for in vitro callus establishment and growth (Shekhawat and Shekhawat 2011; White 1954), while some of their components may suppress the production of certain secondary metabolites in Boraginaceae plants (Koca et al. 2012). For instance, ammonia, manganese, iodate, glycine and thiamine hydrochloride, as well as Fe²⁺ and 2,4-D have been reported to perform inhibitory effects on shikonin production in *L. erythrorhizon* (Fujita et al. 1981; Koca et al. 2012; Mizukami et al. 1977). Besides, plant secondary metabolism might be accelerated when the plant faces suboptimal growth conditions. Like most other secondary metabolites, biochemical pathway of shikonin formation is tightly regulated and the activity of many important enzymes such as phenylalanine ammonia lyase (PAL) and 3-hydroxy-3-methylglutaryl-CoA-reductase (HMGR) can be affected by the composition of growth media and environmental conditions (Gaisser and Hide 1996; Wu et al. 2009; Yazaki et al. 1999). Understanding of the exact mechanisms controlling this complex biochemical pathway needs more detailed experiments.

Here we showed that the optimum levels of IAA and BAP for callus growth and/or shikonin production were different in White, B5 and M9 growth media. Callus growth is achieved by coordinated cell division and cell expansion, in which plant growth regulators, such as auxin and cytokinin, play crucial roles (Davies 2013) in a species-specific and environmentally dependent manner (De-meulenaere and Beeckman 2014; Ljung 2013). On the other hand, shikonin production depends on the activation of secondary metabolism, mediated by complex networks. In spite of many published reports on optimization of plant growth media, there is still no certainty about the proper concentration of each nutrient and plant growth regulator for the formation of various secondary metabolites in

Boraginaceae plants. For example, Mizukami et al. (1977) suggested 10⁻⁶ M IAA and 10⁻⁵ M kinetin for shikonin production in *L. erythrorhizon* cell suspension culture, while Fujita et al. (1981) reported that 10⁻⁵ M kinetin inhibited shikonin production in the same plant. A part of this discrepancy comes from still unresolved crosstalk between various plant growth regulators as well as between plant growth regulators and environmental stimuli (Davies 2013). For instance, a complex interaction between IAA and BAP with brassinosteroids (Yang et al. 1999) or with methyl jasmonate (Ding et al. 2004) in the growth and shikonin formation in cultured *O. paniculatum* cells has been documented. Wu et al. (2009) reported a positive effect of nitric oxide on shikonin production, possibly through methyl jasmonate and ethylene accumulation. A widely accepted scenario is that cell growth and proliferation favor optimal environmental conditions, while secondary metabolite formation favors suboptimal growth conditions. Meanwhile, various stress factors including nutrient starvation, accumulation of toxic compounds, or mechanical damage, which are common in most tissue culture practices, activate secondary metabolism in a species-specific manner (White 1934). Although, the effects of some organic and inorganic compounds on gene expression of key enzymes involved in shikonin formation (PAL and HMGR) have recently been studied (Gupta et al. 2014; Malik et al. 2016; Wu et al. 2009), it seems that elucidation of this complex metabolic pathway requires genome wide microarray analysis and systems biology approaches.

Conclusion

In this study, the RGR of *O. dichroantha* callus and shikonin production in three growth media under various concentrations of IAA and BAP was optimized, using a response surface methodology. After careful statistical analysis, mathematical models were generated that could predict the best concentrations of IAA and BAP for maximum callus growth and shikonin production. According to these analyses, while the B5 medium was better for callus growth, it was not very suitable for shikonin production, such that only 35.96 mg g⁻¹ shikonin could be produced at the optimum conditions. The M9 medium was the best medium for shikonin production (76.83 mg g⁻¹) but it was not suitable for callus growth. The White medium, however, was reasonably suitable for both callus growth and shikonin production, which was predicted to produce 50.64 mg g⁻¹ shikonin at optimum conditions, with the highest observed desirability coefficient (0.903). These data are useful for large scale in vitro propagation of *O. dichroantha* for production of its valuable secondary metabolites.

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Authors contribution MBBN designed the experiment, analyzed the data and wrote the manuscript. TN performed the experiments.

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