Research Article

Flavonoids from cell suspension culture of *Ocimum tenuiflorum* and its enhancement using response surface methodology

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ABSTRACT

Ocimum tenuiflorum (Tulsi) is an aromatic shrub belonging to the basil family Lamiaceae and has been used in Indian traditional medicine. Various characteristics such as its antioxidant, anticancer, antiviral, antiallergic, antimicrobial, and anti-inflammatory activities are major reason to produce the phytochemicals *in vitro*. The current paper studied the enhancement of flavonoid in the plant cell suspension culture of *O. tenuiflorum*. Callus was initiated from leaf explant under optimized treatment of plant growth regulators. Effect of major factors such as precursors and elicitors on the synthesis of flavonoid in the suspension culture was studied. All the optimization studies were carried out with the help of Box–Behnken design of response surface methodology. Results indicated optimization of elicitors and precursors resulted in 2-fold increase in flavonoid synthesis. The generated results can be employed after the validation in upscaled operations for flavonoid production.

KEY WORDS: Elicitor, Flavonoid, *Ocimum tenuiflorum*, Optimization, Precursors

INTRODUCTION

Plants are the sources of innumerable natural products which have proved to be useful as therapeutics, flavoring agents, dyes, and perfumes. These phytochemicals are secondary metabolites which plants produce in its defense mechanism. These metabolites have been widely used since centuries by humankind. Natural products can be isolated from intact plants or can be chemically synthesized. These secondary metabolites are extremely low in amount in plants, as low as 1% of total carbon.[1] The yield is dependent on uncontrollable factors such as climatic conditions or soil conditions. Hence, its isolation from plants is not a good choice in commercialization. The growing demand for natural products and health aspirations has made focus on *in vitro* plant-based processes as potential sources for phytochemical products. Biotechnological approaches to industrial production have proven to be a promising alternative to the traditional agricultural methods in obtaining bioactive metabolites. Strain improvement and media optimization techniques could be carried out to increase the productivity. Interfering compounds in the field-grown intact plant can be eliminated in cell; tissue cultures and cell cultures can be a prospective source of distinct standard phytochemicals in required volumes, and plant cell can perform stereo- and regio-specific biotransformations for the production of compounds even from cheap precursors.[2]

Flavonoids, a group of polyphenolic compound, are of great significance due to its various biological and pharmaceutical activities. The chemical synthesis of flavonoids is difficult and unfeasible in a commercial scale as its structures are complicated with multiple chiral centers. *Ocimum* sp., commonly known as sweet basil, is an annual herb, with important medicinal and culinary properties. This plant is well known for its diverse pharmacological effects and has been extensively a part of traditional medicine for the cure of various ailments. Grayer *et al*.[3] observed that enriched flavonoids extract of *Ocimum* sp. showed strong biological activities and are responsible for the pharmaceutical and biological effects of the plant.

Due to the significant biological activities of flavonoids and their low concentration in plant tissues, researches have sought out alternative ways like cell culture

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techniques to improve the production of flavonoids. Plant cell cultures have a greater rate of metabolism compared to differentiated plants and have shorter biosynthetic cycles.[4] This study deals with enhanced production of flavonoid in cell suspension culture of *Ocimum tenuiflorum*. The strategies followed are optimization of precursor and elicitors in suspension culture for flavonoid production.

MATERIALS AND METHODS

Initiation of Callus

Half strength Murashige and Skoog (MS) (HiMedia, India) medium was prepared with 30% (w/v) sucrose and 0.08% (w/v) agar; pH of 5.7 ± 0.1was maintained and sterilized at 121°C, 15 psi for 15 min for callus induction.

Fresh and young leaves were collected from Herbal Garden (Karunya Institute of Technology and Sciences, Coimbatore) in a clean tissue culture bottle covered with muslin cloth and washed under tap water for 20 min before the surface sterilization. Leaves were then immersed in Tween 20 solution for 10 min with continuous shaking and washed with distilled water several times to remove all traces of Tween. In laminar airflow chamber, the leaves were treated with 0.1% mercuric chloride solution for 2 min followed by rinsing in double-distilled water until all traces were removed. Sterilized young leaves were cut into explants of 0.5 cm^2 in size and inoculated in the sterile medium aseptically. All the cultures were maintained at 25 ± 2°C, 16 h light, and 8 h dark with white fluorescent tubes of the intensity of 1000 lux in the culture room.[5]

Optimization

To examine the relationship between chosen factors (precursors and elicitors) and responses (flavonoid production) and to optimize the production of flavonoids in cell suspension culture, an orthogonal model was used. The medium composition was according to the experimental design [Table 1]. Box–Behnken design (BBD) was developed using Minitab (Minitab® 18.1) using three factors. The factor's levels were allocated into three categories: Low (−1), medium (0), and high (1). Validation experiments were also carried out to verify the validity and the accuracy of the models.

Establishment of Cell Suspension Culture

Half strength sterile liquid MS media were prepared with a pH 5.7 ± 0.1. Friable callus was transferred to the liquid media and the conical flasks were then maintained in incubator shaker at 25°C at 120 rpm. The cell suspension culture was subcultured every 14th day by replacing the medium with fresh medium to avoid accumulation of phenolic compounds.[6]

Precursor Feeding Experiments

Stock solutions of the precursors, phenylalanine, proline, and tyrosine, at 1 g/ml concentration were prepared and filter sterilized using 0.2 µm membrane. A 15 experimental run BBD was used for fitting the second-order response surface. BBD consisted of three factors at three levels, i.e., phenylalanine (1, 3, and 5 ppm), proline (2, 4, and 6 ppm), and tyrosine (1, 3, and 5 ppm). The precursors were added to the culture bottle aseptically according to the BBD in Table 1.

Elicitor Treatment

Stock solutions of abiotic elicitors $AgNO_3$ (1 mM), CuSO4 (1 mM), and salicylic acid (1 mM) were prepared and filter sterilized and were added according to the BBD, Table 1. To the freshly cultured suspension cell, elicitor treatment was done on the 16th day of incubation and flavonoid estimation was done after 72 h.

Extraction of Flavonoid

The cells were harvested by vacuum filtration (0.2 µm) for flavonoid estimation and washed with distilled water twice. After drying the cells in hot air oven at 60°C, about 0.5 g of sample extracted with 10 mL of 80% (v/v) methanol by sonication.[7] After centrifugation at 10,000 revolutions per minute (rpm), flavonoid was estimated by aluminum (III) chloride assay.

Estimation of Total Flavonoid

Aluminum (III) chloride colorimetric assay[8] was carried out to estimate the total flavonoid content in cells of *O. tenuiflorum*. Supernatant (1 ml) was diluted with 4 mL of deionized water to which, 10% (w/v) $NaNO_2$ (0.3 ml) was added and incubated for 5 min. Then, 10% (w/v) $AlCl_3$ (0.3 ml) was added and after 1 min of reaction, 1 M NaOH (2 ml) was added followed by the addition of deionized water to make the reaction mixture volume 10 mL. Then, mixtures were shaken well and the absorbance values of sample were measured using ultraviolet-visible spectrophotometer at 510 nm. The supernatant replaced with 80% (v/v) methanol (1 ml) served as blank. Aliquots of quercetin from 0.2 to 1 g/L were used to construct standard curve.

RESULTS AND DISCUSSION

Establishment of Callus and Suspension Culture

Friable calli obtained after several subculturing of the induced calli and were subsequently used to establish suspension culture. The stabilized suspension culture was obtained after four subculturing of 2 weeks interval.

Table 1: Box behnken Design for experiments and experimental and predicted responses

Run No	A	B	C	Effect of precursors		Effect of Elicitors	
				Flavonoid (mg/g)		Flavonoid (mg/g)	
				Actual response	Predicted response	Actual response	Predicted response
1	1	0	1	29	28.89	53.33	48.85
2	0	-1	1	17.66	18.84	40	31.78
3	-1	0	-1	12.74	11.21	26.66	23.23
4	1	0	-1	23	24.35	13.33	12.81
5	0	0	0	48.06	42.98	40	39.21
6	0	0	0	39.33	42.98	40	39.21
7	0	1	1	26.86	25.52	40	49.8
8	1	-1	0	21.24	18.55	26.66	37.01
9	-1	1	0	7.88	10.57	40	38.89
10	0	-1	-1	12.81	15.82	26.66	22.3
11	1	1	0	27.53	28.99	53.33	49.33
12	-1	0	1	15.45	15.75	13.33	13.75
13	0	0	0	41.53	42.98	40	39.21
14	0	1	-1	22.26	19.46	26.66	32.72
15	-1	-1	0	12.14	10.69	13.33	22.77

(Uncoded values: For Effect of precursors: A: Phenylalanine (mg/L): 1, 3, 5;B: Proline (mg/L):2, 4, 6; C: Tyrosine (mg/L):1, 3, 5 For Effect of elicitors: A: AgNO3 (mM): 0.05, 0.5, 5; B: CuSO4 (mM): 0 0.002, 0.004, 0.008; C: Salicylic acid (mM): 0.05, 0.5, 1.5.)

Optimization by BBD

The second-order polynomial equation was obtained and the interactive effects of the factors were presumed by standard analysis of variance (ANOVA), regression coefficient, F values, and *P* values. The interactions between the two variables and optimum levels can be inferred by considering graphical representations of surface plots.[9] When there are well-defined optimum values for the factors, the response surface will have convex surfaces. When the surfaces are moderately symmetrical and flat near the optimum, the optimized values may not vary from the single variable conditions.[10] Table 1 summarizes the mean values of triplicates obtained from the experimental values. Actual responses are the experimental values and predicted responses are according to the models developed.

Effect of Precursors on Flavonoid Production

Precursor feeding has resulted in an increase in flavonoid production. The observed and predicted responses are given in Table 1. Highest production was observed when the combination of phenylalanine 3 mg/L, proline 4 mg/L, and tyrosine 3 mg/L was fed and lowest production was observed when the combination of phenylalanine 1 mg/L, proline 6 mg/L, and tyrosine 3 mg/L was fed. It is expected that differential biosynthesis of sometimes very complex molecular structures might involve in the regulation of the supply of precursors influencing rate-limiting step for carbon flow through shikimate pathway.[11] Phenylalanine, an upstream metabolic precursor through phenylpropanoid pathway, is a precursor for isoflavones and flavonoids.

The model equation predicted between flavonoid produced and the different precursors fed is obtained as follows:

Flavonoid (mg/ml) = 42.98 + 6.57 Phenylalanine + 2.58 Proline + 2.27 Tyrosine − 12.82 Phenylalanine * Phenylalanine − 12.96 Proline * Proline − 10.11 Tyrosine * Tyrosine + 2.64 Phenylalanine * Proline + 0.82 Phenylalanine * Tyrosine − 0.06 Proline* Tyrosine

The model is significant as $P = 0.006$ and has an insignificant lack of fit ($P = 0.670$). The model terms for phenylalanine, phenylalanine^2, proline^2, and tyrosine^2 are significant with $P < 0.5$ and other terms are insignificant. R^2 and adjusted R^2 represent the proportion of variation in the response that is explained by the model. R^2 (R-Sq) describes the amount of variation in the observed responses that are explained by the model. For the data, 95.66% of the variation in flavonoid production is explained by model. Since R^2 > 90% (goodness of fit), the system is fit. Predicted R^2 reflects how well the model will predict future data. For the data, the predicted R^2 is 64.23%. Adjusted R^2 is a modified R^2 that has been adjusted for the number of terms in the model. For the data, adjusted R^2 is 88.63%. The surface plots [Figure 1a-c] for studying the interaction between the factors are simple and a moderately significant interaction is represented. As the concentration of precursors increased, flavonoid yield also increased and further increased in the precursor reduced the response. Optimum values were determined with optimizer to maximize the yield (44.18 mg/g) as 3.54 mg/l phenylalanine, 4.26 mg/l proline, and 3.22 mg/l tyrosine and evaluated by conducting the experiments with a yield of 43 mg/g which was close to the model predicted.

Phenylalanine has a significant effect on flavonoid production and this is in accordance with the previous reports.[12] It increased metabolic flux through phenyl proponoid biosynthetic pathway. Whereas, proline

treatment on callus of *Hydrocotyle bonariensis* showed higher biomass yield but did not significantly increase flavonoid production. Masoumian *et al.*[13] concluded that the concentration higher than 4 mg/l proline inhibited flavonoid production and flavonoid yield in *Hydrocotyle bonariensis* callus was not significantly altered with glutamine treatment except 1 mg/ml which gave highest yield. The treatments resulted in considerable increase in biomass growth. Addition of 40 mg/l tyrosine in Pegaga cell suspension culture during inoculation had produced 3.2-fold increased flavonoid content in cell. Precursors have induced stress to cell by suppressing growth and promoting flavonoid biosynthesis.[14] Margna *et al.*[15] observed that the simultaneous administration of phenylalanine reduced the uptake of tyrosine, but a reciprocal effect of tyrosine on absorption of phenylalanine was not detected.

Effect of Elicitor on the Flavonoid Production

The flavonoid production is significantly influenced by $CuSO_4$, salicylic acid, and interaction between $AgNO_3$ and salicylic acid. The elicitor treatment on the 16th day has improved the flavonoid production in suspension culture. The actual response and the predicted responses are given in Table 1.

The regression coefficients of the second-order equations have been obtained using experimental data as follows:

Flavonoid (mg/ml) = 39.21 + 6.17$AgNO_3$ + 7.11 $CuSO_4$ + 6.64salicylic acid − 5.85$(AgNO_3)^2$ + 3.64$(CuSO_4)^2$ − 8.70(salicylic acid)^2 − 0.95($AgNO_3$* $CuSO_4$) + 11.38($AgNO_3$* salicylic acid) + 1.90($CuSO_4$* salicylic acid)

The model is significant (P = 0.05) and the "lack of fit" is insignificant (P = 0.06). The regression coefficient R^2 is 89.14% and adjusted R^2 is 69.6% in the model; $CuSO_4$, salicylic acid, and ($AgNO_3$* salicylic acid) are significant with $P < 0.05$. In the surface plots [Figure 2a-c], the interaction between the factors could be seen as for $AgNO_3$ and $CuSO_4$, as $AgNO_3$ increases, flavonoid also increases and then decreases but for $CuSO_4$, flavonoid increases as $CuSO_4$ increases. Other combinations also showed similar observation. The flavonoid content increases initially to reach a maximum value and then decreases. The optimum elicitor concentration to maximize the flavonoid production (60.55 mg/g) was found with response optimizer as 0.5 mM of $AgNO_3$, 0.002 mM of $CuSO_4$, and 0.5 mM of salicylic acid and was validated by experiment as 58 mg/g, which is close to the model predicted.

Stress response in plants results in reversible phosphorylation, ion fluxes, salicylic acid, or oxylipins such as jasmonic acid, ethylene, reactive oxygen species, transcription factors, and promoter elements.[16] Eicitors act by induction or removal of repression on the biosynthesis of secondary metabolites. Abiotic chemical agents stimulate secondary metabolism and disruption of cells leads to production in large quantity.[17] Cupric sulfate addition released large amount of berberine as it stimulates and permeabilize cells to release product; however, other heavy metals did not act in the same way.[18] Copper also stimulated the production of shikonin derivatives; however, the yield became constant for copper above 0.8 M.[19]

Salicylic acid induces gene regulation related to the biosynthesis of secondary metabolites in plants.[20]

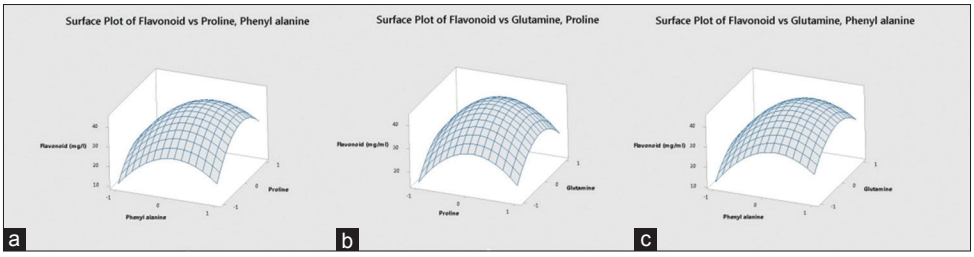

Figure 1: (a-c) Surfacet plots for precursor

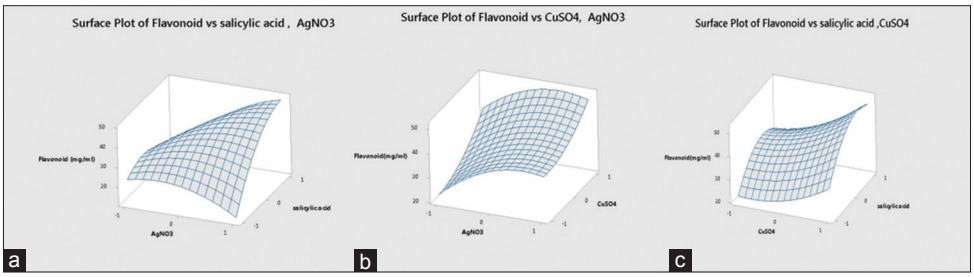

Figure 2: (a-c) Surface plots for elicitor

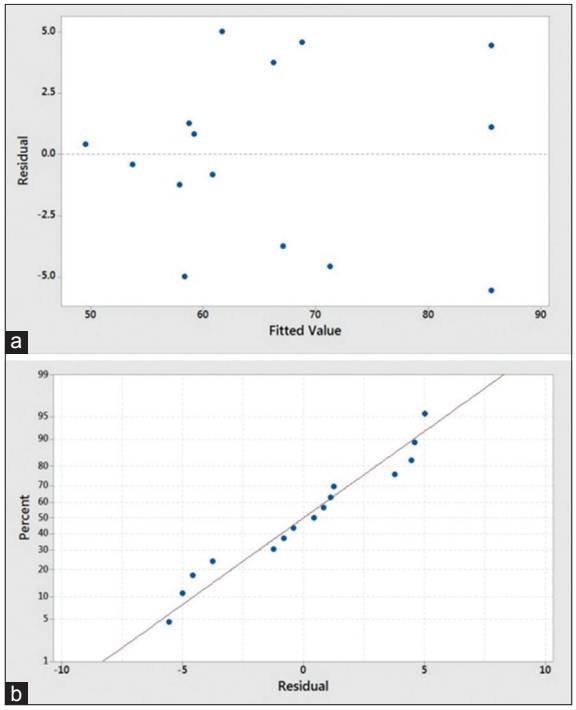

Figure 3: (a) Residual versus fitted values for plant growth regulator (PGR) on callus induction. (b) Normal probability plot for PGR on callus induction

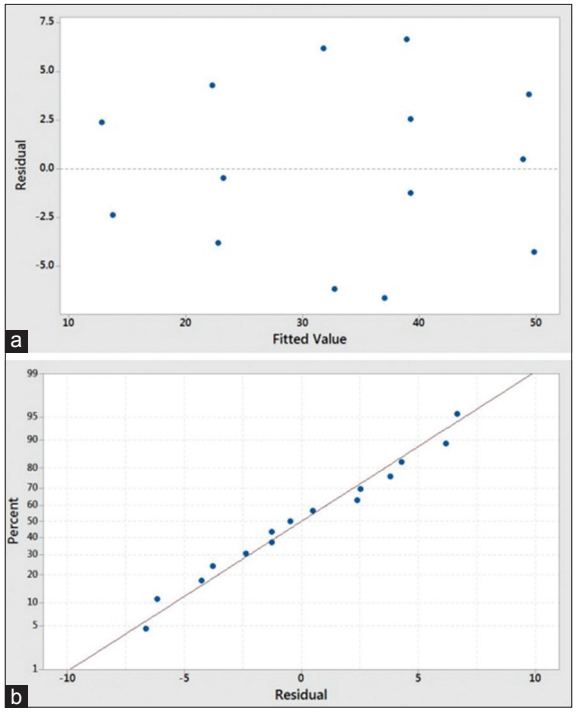

Figure 5: (a) Residual versus fitted values for elicitors on flavonoid production. (b) Normal probability plot for elicitors on flavonoid production

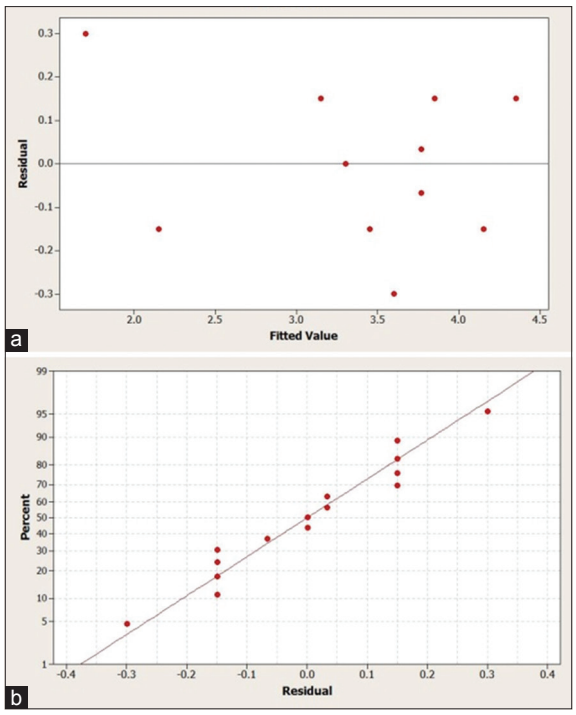

Figure 4: (a) Residual versus fitted values for precursors on flavonoid production, (b) Normal probability plot for precursors on flavonoid production

Goyal and Ramawat[21] optimized 20 µM of salicylic acid for 48 h of treatment as most favorable in isoflavonoid production in *Pueraria tuberose* cell suspension culture. Mendhulkar and Vakil[22] reported that increased concentration of salicylic acid increased total flavonoid accumulation. Optimal concentration was found to be 1.5 mM for 48 h of treatment and resulted in 1.14-fold increase as compared to the control.

In plant cells, Ag+ has key role in secondary metabolite pathways, wherein it induces significant enzymes in jasmonic acid pathway and inhibits ethylene signal transduction pathway.[23,24] Zhang *et al*.[25] observed in root cultures of *Salvia miltiorrhiza*, Ag+ induced production of diterpenoids. Similarly, Ag+ reported to increase yield of scopolamine and hyoscyamine in the roots culture.[26] Methyl jasmonate treatment in cell cultures inhibited the activities of catalase and stimulated phenylalanine ammonia lyase which led to increased flavonoid production.[7]

Diagnostics of the Model

The competence of a model fit is assessed with residual analysis.[27] Residual is the difference between the actual values and predicted values based on the model. The model equation does not include the residuals. The residual study is carried out by graphical methods. If the selected model is acceptable, the residuals are distributed equally on a horizontal axis of graph. Figures 3a, 4a, and 5a show random distribution of the residuals as function of the model values, so homoscedasticity hypothesis is proved. In Figures 3b, 4b, and 5b, residuals are independent of each other and are normally distributed. In this study, the satisfactory normal distribution confirms that

the regression model predicted has utilized all the observations from the experiments.

CONCLUSION

The flavonoids have become one of the most important secondary metabolites for commercialization. Flavonoids are important components in most of the bioactive drugs and in drug formulation. Thus, scaling up of the production of flavonoid is necessity and the methods used in industries for isolation of flavonoid from plants are a tedious work and a lot of resources go into purification to get the final product. Thus, by enhancing flavonoid production in cell suspension culture, isolation of flavonoid from plant cells can be more efficient and precise. Response surface methodology has been proved to be suitable for optimizing plant growth regulators, elicitors, and precursors in plant tissue culture. The growth regulators have reduced the time for callusing. Flavonoid production was increased 2.15-fold by precursor feeding and 2.8-fold by elicitor treatment. This methodology can be utilized in large-scale production of flavonoids from *O. tenuiflorum* suspension culture.

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