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Effect of Salicylic Acid on the Activity of PAL and PHB Geranyltransferase and Shikonin Derivatives Production in Cell Suspension Cultures of *Arnebia euchroma* (Royle) Johnst—a Medicinally Important Plant Species

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Abstract Cell suspension cultures of *Arnebia euchroma* were established from the friable callus on liquid Murashige and Skoog medium supplemented with 6-benzylaminopurine (10.0 μM) and indole-3-butyric acid (5.0 μM). Salicylic acid was used to study its effect on the enzymes which participate in shikonin biosynthesis with respect to metabolite (shikonin) content in the cell suspension culture of *A. euchroma*. In our study, phenylalanine ammonia lyase and PHB geranyltransferase were selected from the entire biosynthetic pathway. Results showed that phenylalanine ammonia lyase is responsible for growth and PHB geranyltransferase for metabolite production. Salicylic acid exhibited an inverse relationship with the metabolite content (shikonin); salicylic acid (100 μM) completely inhibited shikonin biosynthesis. The results presented in the current study can be successfully employed for the metabolic engineering of its biosynthetic pathway for the enhancement of shikonin, which will not only help in meeting its industrial demand but also lead to the conservation of species in its natural habitat.

Keywords *Arnebia euchroma* · Boraginaceae · Cell suspension culture · Shikonin derivatives · Secondary metabolites · PAL · PHB geranyltransferase

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Introduction

Shikonin is a plant secondary metabolite naturally occurring in roots of plants belonging to the family Boraginaceae such as *Alkana tinctoria*, *Arnebia gutata*, *Arnebia hispidissima*, *Arnebia benthamii*, *Arnebia nobilis*, *Lithospermum erythrorhizon*, and so on. These compounds are 1,4-naphthaquinone derivatives having various useful properties like anti-fungal [1], anti-inflammatory [2], anti-topoisomerase-I [3], anti-HIV-I activity [4], anti-bacterial [5], anti-tumor activity [6,7], anti-allergic, anti-hydropic, anti-neoplastic, antipyretic and wound healing [8], curing of ulcers and burnt skin, and as a dyestuff for food, fabric, and cosmetic. Owing to these properties, genus *Arnebia* is over-exploited and therefore it is placed under the category of critically endangered species [9,10]. Shikonin is the first plant secondary metabolite produced commercially from *L. erythrorhizon* under in vitro conditions [11]. The cost of production and geographical limitation of *L. erythrorhizon* forced the researchers to find out other methods or sources to deal with the increasing demands of shikonin in the industries. *A. euchroma* (Royle) Johnst is one of the medicinal plant species found in the Northwestern Himalayas that also produces shikonin in the roots (Fig. 1). Shikonin is biosynthetically derived from two precursors—*p*-hydroxybenzoic acid (PHB) (obtained via phenylpropanoid pathway/shikimate pathway) and geranyl diphosphate (GPP) (obtained via mevalonate pathway) [12] (Fig. 2). Phenylalanine ammonia lyase (PAL) converts phenylalanine to cinnamic acid in a phenylpropanoid pathway, which leads to the formation of the first precursor, *p*-hydroxybenzoic acid, of shikonin biosynthesis. Similarly, PHB geranyltransferase forms an intermediate compound—*m*-geranyl-*p*-hydroxybenzoic acid (GBA) via condensation of GPP and PHB [11]. It is generally acknowledged that the main problem in enhancing the production of metabolites to higher levels has been the lack of basic knowledge on biosynthesis, transport, and accumulation of metabolites. In this regard, the current study is aimed to enhance the secondary metabolite production through elicitors and their effect on the activity of enzymes which participate in the metabolic pathways, i.e., PAL and PHB geranyltransferase in suspension culture of *A. euchroma*.

Materials and Methods

Plant Material and Sterilization

Leaf explants excised from the young plants growing in polyhouse were washed in running tap water for 5–10 min. The explants (5–7 cm) were rinsed in distilled water having one to two

Fig. 1 **a** *A. euchroma* in its natural habitat. **b** Roots of *A. euchroma*

drops of liquid detergent (Teepol or Tween-20) for 10 min, followed by 5 min of washing with distilled water. The leaves were then surface-sterilized with bavistin (0.02 %*w*/*v*) and streptomycin (0.02 %*w*/*v*) for 10 min, followed by washing with distilled water thrice. The explants were then disinfected with 70 % ethyl alcohol treatment for 30–40 s. The leaves were then treated with 0.01 % $HgCl_2$ (having one to two drops of Tween-20) for 5 min, followed by five washings with sterile distilled water inside the laminar airflow.

Establishment of In Vitro Callus Culture

Culture Media

Murashige and Skoog (MS) medium was used for induction of callus from leaf explant (1.0–1.5 cm). Sucrose (3 %; *w*/*v*) was added as a carbohydrate source, and agar (0.8 %; *w*/*v*) was incorporated as a gelling agent. 6-Benzylaminopurine (BAP) (10 μM) and indole-3-butyric acid (IBA) (5 μM) were used as plant growth regulators for callus induction. The pH of the media was adjusted to 5.75 prior to autoclaving. The media was autoclaved for 20 min at 15 psi and 121 °C temperature. Culture vessels (250-ml Erlenmeyer conical glass flasks) were used, containing 50 ml medium.

Establishment of Cell Suspension Culture

The cell suspension cultures (M9 Medium) were established using MS medium supplemented with BAP (10 μM) and IBA (5 μM) [8]. Sucrose (3 %*w*/*v*) was added as carbohydrate source. The pH of media was adjusted to 5.75 prior to autoclaving. Erlenmeyer glass conical flasks (250 ml) were used as culture vessels, containing 50 ml of medium. Different concentrations of salicylic acid (SA) (Sigma, St. Louis, MO, USA) (10, 20, 50, and 100 μM) were applied to M9 medium of *A. euchroma*. The flasks were covered with aluminum foil to avoid any leakage of media or contamination. The cultures were kept on a shaker set at 100 rpm, 25−2 °C under dark conditions and maintained with regular subculturing at 10-day interval.

Enzyme Assays

Preparation of Cell-Free Extract

Cells (1 g) were suspended in potassium phosphate buffer (3 ml; 0.1 M; pH 6.5) containing DTT (10 mM) and PVPP (0.2 g). The suspension was homogenized and centrifuged at 4 °C, 8,000 rpm for 10 min. Supernatant was obtained and applied to Sephadex G-25 gel filtration column equilibrated with a reaction buffer containing DTT (10 mM). Cell-free extract was obtained and used as crude enzyme for the enzyme assay. Protein content was determined by Bradford method using bovine serum albumin (Sigma, St. Louis, MO, USA) as a standard.

Assay for Phenylalanine Ammonia Lyase Activity

The activity of phenylalanine ammonia lyase was measured according to the method described by Heide et al.[13] with slight modification. The mixture (100 μl) containing enzyme extract (80 μl), sodium borate buffer (0.2 M; pH 8.7), and phenylalanine (2 μM) was incubated for 75 min at 30 °C. The reaction was stopped by adding of tricarboxylic acid (5 N), followed by centrifugation at 4 °C, 8,000 rpm. 4-Methylumbeliferone (50 nM) was used as a standard.

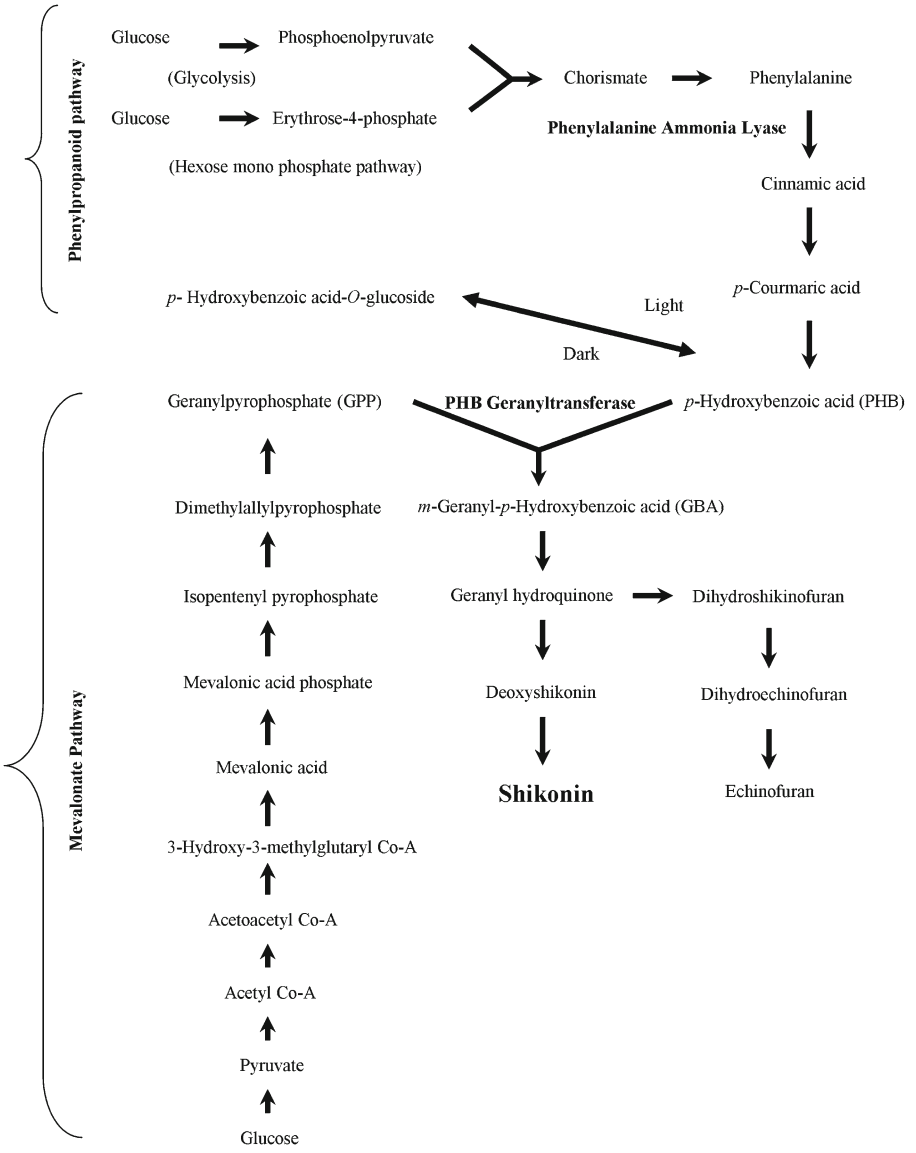

Fig. 2 An outline of biosynthetic pathway of shikonin and its derivatives (adopted from Gaisser and Heide [21])

After centrifugation, PAL activity was determined by measuring the absorbance at 290 nm using a spectrophotometer.

Assay for Para-hydroxybenzoic Acid Geranyltransferase Activity

PHB geranyltransferase activity was measured according to the method of Heide et al.[13]. The mixture (100 μl) containing enzyme extract (80 μl), Tris–HCl buffer (10 μM; pH 7.5), para-hydroxybenzoic acid (0.1 μM), geranylpyrophosphate (0.2 μM), and $MgCl_2$ was incubated for 60 min at 30 °C. The reaction was stopped by adding 5 μl ethyl acetate, followed by

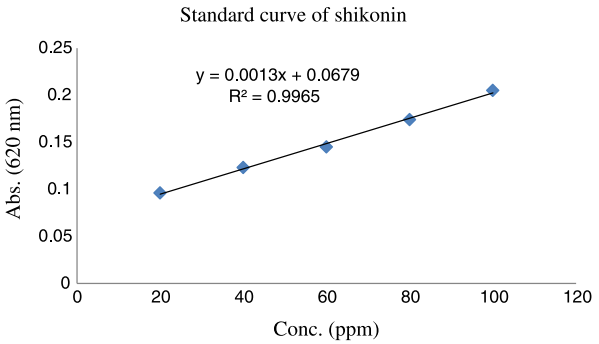

Fig. 3 Standard curve of shikonin

centrifugation at 4 °C, 8,000 rpm. Diethylstilbestrol (20 nM) was used as a standard. After centrifugation, PGT activity was determined by measuring the absorbance at 254 nm using a spectrophotometer.

Spectrophotometric Analysis of Shikonin Derivatives

The production of shikonin in M9 medium was measured spectrophotometrically. One-gram sample was processed with pestle–mortar and 2 ml of iso-amyl alcohol was added to it, followed by gentle maceration for 2 min. Then, 500 μl of iso-amyl alcohol was placed in a fresh micro-centrifuge tube to which 1 ml of freshly prepared KOH (2.5 %) was added, followed by gentle shaking for 5 min [14]. The mixture was then allowed to stand for 10 min so that the aqueous and the organic layers got separated. The organic layer was discarded and the blue-colored lower layer (500 μl) was used to record absorbance at 620 nm [15]. The measured OD value was calculated by using the standard curve of shikonin (Fig. 3).

Statistical Analysis

Three replicates were used for each treatment, and each experiment was repeated thrice. Analysis of variance (ANOVA) was performed, and the mean of the results was compared by Duncan's multiple-range tests at 5 % significance level.

Results and Discussion

Effect of Salicylic Acid on Cell Growth and pH

In the present study, cell growth was observed to be greatly influenced by salicylic acid in M9 medium. Cell biomass was found highest in the control than in salicylic acid treatments, which shows an inverse relation between cell biomass and salicylic acid concentration (Fig. 4). The *A. euchroma* cells were transferred to M9 medium under aseptic conditions with different salicylic acid treatments to observe its effect on biomass yield. Results revealed that cell biomass was decreased until the 2nd day in all the SA treatments and control; thereafter, it remains constant until the 4th day in the cultivation period. From day 4, a continuous increase in cell biomass of medium was observed, except in 100 and 50 μM SA treatments until the completion of the cultivation period. Maximum biomass was found on the 10th day in control (7.07 g FW/50 ml) as compared to 100 μM SA (3.5 g FW/50 ml) (Table. 1, Table No. 2).

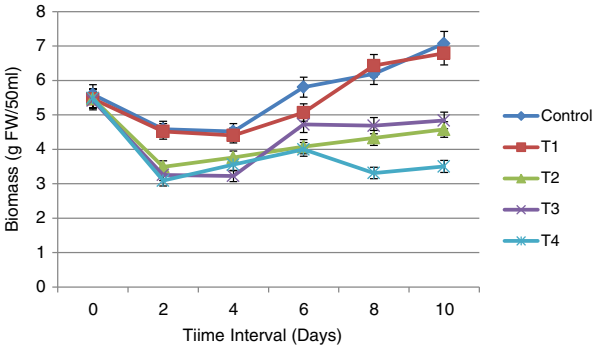

Fig. 4 Effect of salicylic acid on the cell biomass (g FW/50 ml) (where T1=10 μM; T2=20 μM; T3=50 μM; T4=100 μM) (±SD)

Similarly, the pH of the medium was found to have a pronounced effect on cell biomass. Cell growth is directly influenced by the nutritional composition of the medium, but the uptake of nutrient is mainly influenced by the pH of the medium. The various biochemical reactions are occurring at the cellular level, influenced by changes in pH due to the specificity of pH-dependent enzymes. The results also revealed that PAL requires the optimum pH ranges from 5.30 to 5.55 for its activity (Fig. 5). In our experimental conditions, a slightly decreasing trend of pH was observed until the 4th day; thereafter, the pH continued to increase until the 8th day of the culture period. Thereafter, a sharp decrease was observed until completion of cultivation.

Effect of Salicylic Acid on Phenylalanine Ammonia Lyase Activity

PAL is an inducible enzyme enhanced by a wide variety of physico-chemical factors. PAL is a key enzyme which participates in the shikimate pathway or phenylpropanoid pathway, leading to the formation of cinnamic acid by the conversion of phenylalanine [16]. SA has been used as a signaling molecule or elicitor in the plant cell culture. In our study, SA was used to evaluate its effect on the activity of phenylalanine ammonia lyase, a key enzyme in shikonin biosynthesis. It was observed that the PAL activity of the control was almost higher in all the days of sampling as compared to the other treatments except in treatment 2 (20 μM) and 4 (100 μM) in day 0. On the 10th day, the PAL activity in 10 μM SA treatment is close to that of control, which means that this concentration does not inhibit the activity of PAL (Fig. 6). From our experimental observations, it was concluded that the activity of PAL does not show a direct relationship with the shikonin content, which is consistent with the results obtained by Baranek et al.[17] who also showed that PAL activity does not contribute to shikonin content. Similarly, the same kind of result was obtained by Macdonald and D'cunha [16], who also illustrated that PAL does not necessarily contribute to the production of pigments; it is possible that PAL might be directed to the production of protectants against biotic and abiotic stress and mechanical support (lignin). However, the PAL activity shows a direct relationship

Table 1 Different salicylic acid concentrations used in the suspension culture of *A. euchroma*

Suspension no.	Treatments (μM)	Salicylic acid (μl)	Ethanol (μl)
1	Control	–	–
2	T1 (10)	50	450
3	T2 (20)	100	400
4	T3 (50)	250	250
5	T4 (100)	500	–

Table 2 Effect of salicylic acid on cell growth and pH; ANOVA test shows high significance with Duncan multiple-range test at 5 % (p=0.05). Data shown are the means of three replicates of three cultures in each treatment ± standard error (SE). Data were recorded within 1-week interval

Treatment no.		0 days (mean ± S.E.)	2 days (mean ± S.E.)	4 days (mean ± S.E.)	6 days (mean ± S.E.)	8 days (mean ± S.E.)	10 days (mean ± S.E.)
Control	Biomass (g FW/50 ml)	5.60d±0.38	4.59c±0.43	4.52de±0.05	5.81e±0.08	6.19de±0.08	7.07f±0.25
T1		5.47 cd±0.32	4.52c±0.13	4.41 cd±0.05	5.06 cd±0.02	6.43e±0.06	6.79f±0.14
T2		5.47 cd±0.12	3.49a ±0.15	3.76b±0.08	4.07ab±0.16	4.33b±0.01	4.58b±0.04
T3		5.42 cd±0.03	3.26a±0.00	3.23a±0.07	4.72c±0.02	4.69b±0.10	4.84b±0.13
T4		5.49 cd±0.03	3.09a±0.05	3.56b±0.11	4.00a±0.03	3.31a ±0.02	3.50a±0.03
Control	pH	5.50 cd±0.55	4.81c ±0.39	4.39 cd±0.02	5.10 cd±0.54	5.86 cd±0.42	5.52 cd±0.29
T1		5.38e±0.03	5.96d±0.03	4.78e±0.01	4.58bc±0.06	5.43c±0.05	5.31c±0.16
T2		5.20c±0.23	5.97d±0.02	5.40f±0.25	5.46de±0.03	6.12de±0.06	6.08e±0.04
T3		4.83b±0.13	4.59c±0.03	4.40 cd±0.01	6.52f±0.02	6.25de±0.05	5.87de±0.05
T4		4.22a ±0.11	4.06b±0.09	4.12c±0.04	3.98a±0.01	6.08de±0.02	5.83de±0.06

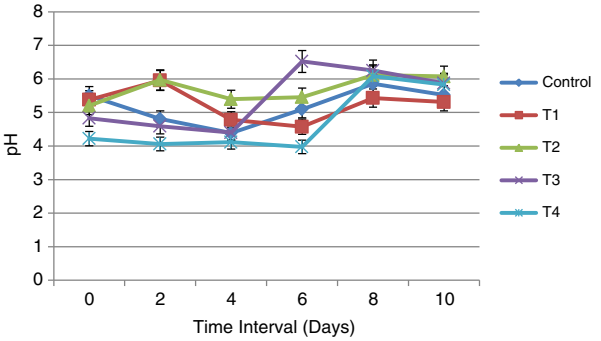

Fig. 5 Effect of salicylic acid on pH (where T1=10 μM; T2=20 μM; T3=50 μM; T4=100 μM) (±SD)

with the cell biomass as was observed in our study, which revealed that PAL participates in cell growth. Highest cell biomass was reached by the control (7.07 g FW/50 ml) as compared to SA treatments (10 μM SA, 6.79 g FW/50 ml; 20 μM SA, 4.5 g FW/50 ml; 50 μM SA, 4.8 g FW/50 ml; 100 μM SA, 3.5 g FW/50 ml). It was concluded that as the activity of PAL increases, cell biomass also rises, which shows its relationship with the cell biomass.

Effect of Salicylic Acid on PHB Geranyltransferase Activity

PHB geranyltransferase is an important enzyme which participates in the conversion of PHB acid and GPP to an intermediate GBA in shikonin biosynthesis [13]. SA has greatly influenced the activity of PHB geranyltransferase in the suspension culture of *A. euchroma*. It was observed that shikonin production was decreased with enhancement in the concentration of SA. Similarly, the activity of PHB geranyltransferase was completely inhibited by SA. Activity in control samples is continuously increasing with the cell culture days (0 days, 0.0101 U/ml; 4th day, 0.0194 U/ml; 10th day, 0.0217 U/ml), while in the SA-treated cells, activity is greatly reduced. This shows the inverse relationship between the SA and PHB geranyltransferase activity (Fig. 7). Our results conclude that this enzyme is one of the key regulatory enzymes in shikonin biosynthesis, which is in agreement with earlier studies' reports [8,18,19]. Similar results were also found by Tabata and Fujita [20] who described the effect of methyl jasmonate, sodium deoxycholate, *n*-octyl-*b*-D-glucopyranoside, CHAPS, and digitonin in the suspension culture of *L. erythrorhizon.*

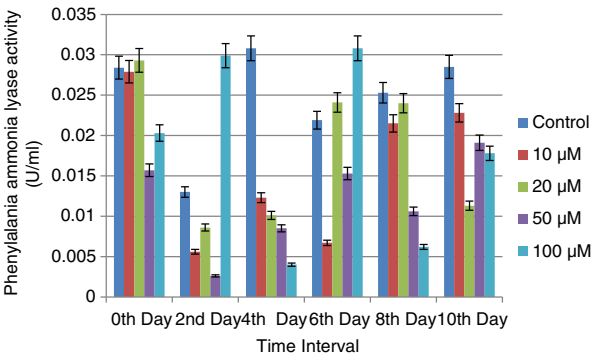

Fig. 6 Effect of salicylic acid on phenylalanine ammonia lyase activity (±SD)

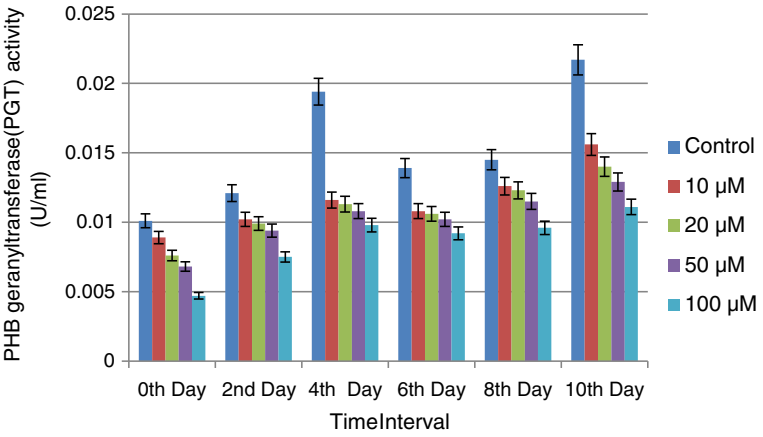

Fig. 7 Effect of salicylic acid on PHB geranyltransferase activity (±SD)

Effect of Salicylic Acid on Pigment (Shikonin and Its Derivatives) Production

In the present study, the maximum production of shikonin was observed within 10 days of cultivation period; thereafter, it declined. The decline was possibly due to cell death or as a result of feedback inhibition caused by the accumulation of pigment. Dark condition was a prerequisite for the biosynthesis of pigment as there was no pigment production in light. This is due to the fact that these compounds are accumulating in the underground part of the plant. The production of shikonin in M9 medium was measured as described by Yazaki et al.[14] using the standard curve of shikonin (Fig. 3). Shikonin content was observed to be highest (527.90±1.42 mg/l) in the control as compared to the salicylic acid treatments (10 μM=136± 2.0 mg/l; 20 μM=105.20±2.6 mg/l; 50 μM=57.38±2.1 mg/l; 100 μM=not detected; Fig. 8) which revealed that salicylic acid inhibits shikonin production in the suspension culture of *A. euchroma* (Fig. 9). This might be due to inhibition of enzymes which participate in shikonin biosynthesis. In the presence of 10 μM SA, the shikonin content reached 136±2.0 mg/l as compared to the 100 μM SA with no production of shikonin, which shows that SA was directly

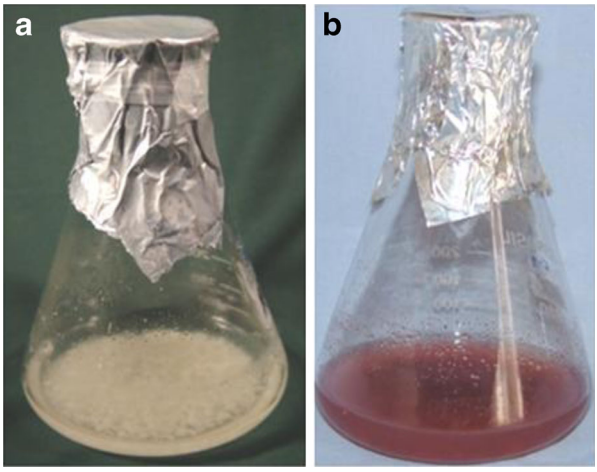

Fig. 8 *A. euchroma* cells. **a** Salicylic acid-treated (100 μM). **b** Control

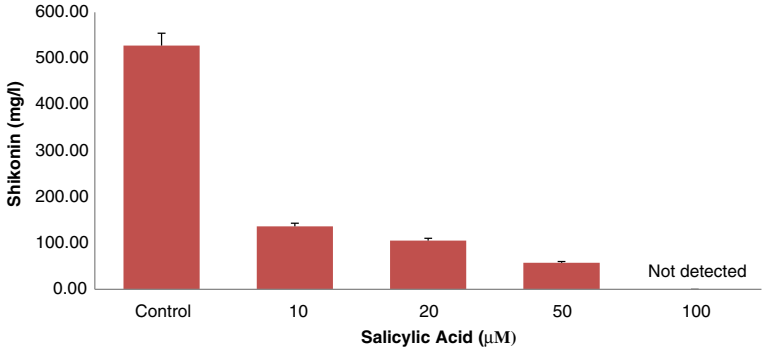

Fig. 9 Effect of salicylic acid on pigment (shikonin) production (±SD)

correlated with shikonin biosynthesis. It was concluded that SA completely inhibits shikonin biosynthesis as it was observed in our experimental conditions. SA was not applied as an elicitor for shikonin biosynthesis as it is used in other plant cell cultures.

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