

# Combined UV-C irradiation and precursor feeding enhances mulberroside A production in *Morus alba* L. cell suspension cultures

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**ABSTRACT:** Mulberroside A is a major bioactive compound produced by *Morus alba* L., a medicinal plant of the family Moraceae that is used in traditional medicines and supplementary health products for various indications. In this study, cell suspension cultures of *M. alba* that produced mulberroside A and other phytochemicals were treated with 20 min UV-C irradiation to investigate the effect of time after UV-C treatment on levels of mulberroside A, oxyresveratrol, resveratrol, and rutin. The amount of mulberroside A produced from cell suspension cultures of *M. alba* that were treated with UV-C was significantly higher than the control. However, there were no significant differences in the levels of oxyresveratrol, resveratrol, and rutin. The effect of UV-C in combination with precursor feeding on phytochemical production of cell suspension cultures of *M. alba* was investigated. L-phenylalanine and L-tyrosine were used as precursors. The addition of 0.05 mM L-phenylalanine 5 d prior to UV-C irradiation doubled the content of mulberroside A in cell suspension cultures of *M. alba* as compared to the non-irradiated and untreated cells. The use of precursor feeding in combination with UV-C irradiation could be an alternative method for the production of mulberroside A in *M. alba*.

**KEYWORDS:** cell suspension culture, elicitor, *Morus alba*, mulberroside A, precursor

## INTRODUCTION

*Morus alba* L. is a dominant species of the genus *Morus*, belonging to the family Moraceae. This plant is widely distributed in the temperate and tropical regions including East and Southeast Asia, Southern Europe, North America, and some parts of Africa [1]. Its leaves are used to feed silkworms in the process of silk production. Its fruits are edible, both fresh and processed. Its root and bark are often included in traditional Chinese medicines. Different parts of *M. alba* are also used in folk remedies in many countries. Several studies have reported pharmacological activities of *M. alba* extracts and their bioactive components, including anti-oxidative [2], anti-inflammatory [3], hypoglycemic [4], anti-hyperlipidemic [5], and anti-viral [6] activities.

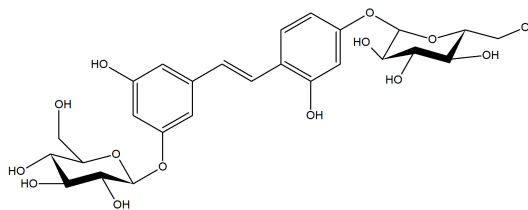


Fig. 1 Chemical structure of mulberroside A.

*M. alba* is a rich source of stilbenoids which are a group of polyphenolic compounds responsible for its various bioactivities. On the other hand, other important polyphenolic compounds such as flavonoids (eriodictyol and homoeriodictyol) were found to be the major active compounds in the stems of *Dendrobium* spp. [7]. Mulberroside A, a glycosylated stilbenoid, is a major component found

in the roots, barks, and stems of *M. alba* (Fig. 1). In addition, aglycone resveratrol and oxyresveratrol, which are abundant in grape vines, are also present in *M. alba*. These stilbenoids are biosynthesized through the phenylpropanoid pathway which involves L-phenylalanine (Phe) and L-tyrosine (Tyr) as their precursors.

Plant cell culture techniques are effective alternatives for the production of plant secondary metabolites. Many successful protocols for the production of high-value bioactive compounds by *in vitro* culture have been established and reported [8]. Various strategies, such as cell growth optimization, immobilization of cells, application of elicitors, and precursor feeding, have been adopted for further yield improvements. These techniques are also effective for the production of stilbenoids in *in vitro* plant cultures [9–11]. Ultraviolet C (UV-C) irradiation is a postharvest treatment used for the improvement of storage potential and also acts as an abiotic elicitor to enhance the biosynthesis of phenolic compounds in vegetables and fruits [12]. Several studies have reported the enhancement of stilbenoid accumulation in grape vine crops and *in vitro* cultures in this way [13–15].

Previously, successful production of mulberroside A in cell suspension culture of *M. alba* [16] and the enhancement of stilbenoid production in an immobilized cell culture of *M. alba* treated with the precursors, Phe and Tyr, have been reported [11]. In this study, we aimed to investigate the effect of UV-C irradiation in combination with precursor feeding on mulberroside A, oxyresveratrol, resveratrol and rutin accumulation in cell suspension cultures of *M. alba*.

## MATERIALS AND METHODS

### Chemicals

Mulberroside A (purity 99.42%) was commercially obtained from Chengdu Biopurity Phytochemicals (Sichuan, China). Oxyresveratrol was isolated from *Artocarpus lakoocha* as previously described [17]. Resveratrol (purity  $\geq 99\%$ ) and rutin (purity  $\geq 95\%$ ) were purchased from Sigma Chemical (St Louis, USA). Phe and Tyr were obtained from Sigma-Aldrich (St Louis, USA). All other chemicals were standard analytical reagent grade commercial products.

### Establishment of *M. alba* cell suspension culture

Cell suspension culture of *M. alba* was established as previously described [16]. Briefly, leaf explants

were cultured on solidified Murashige and Skoog (MS) medium [18] supplemented with 0.1 mg/l thidiazuron (TDZ) and 1 mg/l naphthalene acetic acid (NAA) to initiate callus culture. Leaf-derived calli were transferred to 125 ml flasks containing 30 ml MS medium supplemented with 0.1 mg/l TDZ and 1 mg/l NAA to establish cell suspension culture and maintained on a rotary shaker at 110 rpm in a 16:8 h light/dark cycle at 25 °C.

### UV-C treatment

To study the effect of UV-C on phytochemical production, 21-day-old *M. alba* cell suspension cultures were incubated in dark conditions for 3 d before being transferred to petri dishes and irradiated under a UV-C lamp (254 nm, 15 W, distance 0.6 m, irradiation intensity 36.6 W/m<sup>2</sup>) for 20 min. Cell cultures were then transferred back into the flasks and cultured on a rotary shaker at 110 rpm in a 16:8 h light/dark cycle at 25 °C. Samples were collected for determination of mulberroside A, oxyresveratrol, resveratrol, and rutin initially and 12, 24, 36, and 48 h after UV-C irradiation. Cell cultures without UV-C irradiation were used as controls.

### Precursor feeding

The effect of precursor feeding in combination with UV-C irradiation was determined by adding precursors to the cell suspension cultures prior to UV-C treatment. An amount of 0.05 mM Phe and 0.03 mM Tyr (either alone or in combination) was used as a precursor in this experiment. Precursors were added to *M. alba* cell suspension cultures for either 5 d or 10 d prior to UV-C treatment. For the UV-C treatment, 21-day-old cell suspension cultures were incubated in dark conditions before irradiated under a UV-C lamp, using the same conditions as mentioned above. Samples were collected for quantification of phytochemicals after UV-C irradiation. Cell culture without UV-C irradiation and precursor feeding was used as a control.

### Sample extraction

Harvested samples were dried in a hot air oven at 50 °C for 48 h. Subsequently, 10 mg of dried, powdered samples was extracted with 0.5 ml methanol under sonication. The extraction was repeated four times. Extracted solutions were collected together, evaporated in a water bath at 50 °C, and re-dissolved in 1 ml methanol. Samples were diluted appropriately and subjected to analysis by indirect competitive ELISA and HPLC.

A previous study reported that mulberroside A could also be released from cells into the medium [16]. Therefore, *in vitro* culture media of the cell suspension cultures were collected for analysis of extracellular mulberroside A content. Methanol was added to the media samples to a final concentration of 20% methanol, and they were then serially diluted to the appropriate concentrations and subjected to analysis by indirect competitive ELISA.

#### Quantification of mulberroside A by indirect competitive ELISA

Mulberroside A was determined using the indirect competitive ELISA developed by the research group, as previously reported [19]. Briefly, a 96-well plate was coated with mulberroside A-ovalbumin conjugate and incubated at 37°C for 1 h. The plate was treated with 1% gelatin in phosphate buffer solution to reduce nonspecific absorption. Then, various concentrations of the mulberroside A standard and the extracts prepared in 20% methanol in water were incubated with polyclonal antibody against mulberroside A at 37°C for 1 h. Peroxidase-labeled anti-rabbit IgG antibody was used as a secondary antibody. The absorbance of the color reaction was measured at 405 nm using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) as a substrate and a microplate reader (Model 550 Microplate reader, Biorad Laboratories). All samples were prepared and analyzed in triplicate.

#### Quantification of oxyresveratrol, resveratrol and rutin by HPLC

HPLC analysis to determine oxyresveratrol, resveratrol, and rutin was performed using a PerkinElmer Series 200 LC pump equipped with a PerkinElmer 785A UV/VIS detector. An RP-18 column (LiChro-CART®, 125 × 4 mm, 5 µm particle size; Merck, Germany) was used as the stationary phase. The mobile phase was 20% aqueous acetonitrile containing 0.5% acetic acid. HPLC analysis was performed at a flow rate of 1 ml/min and a detection wavelength of 320 nm. All samples were prepared and analyzed in triplicate.

#### Statistical analysis

The data were expressed as the mean ± standard deviation (SD) from triplicate samples. The difference between samples was tested using a one-way analysis of variance (ANOVA) with a *post-hoc* Tukey's HSD test. Differences at  $p < 0.01$  were considered statistically significant.

## RESULTS

### Effect of UV-C on phytochemical production in cell suspension culture of *M. alba*

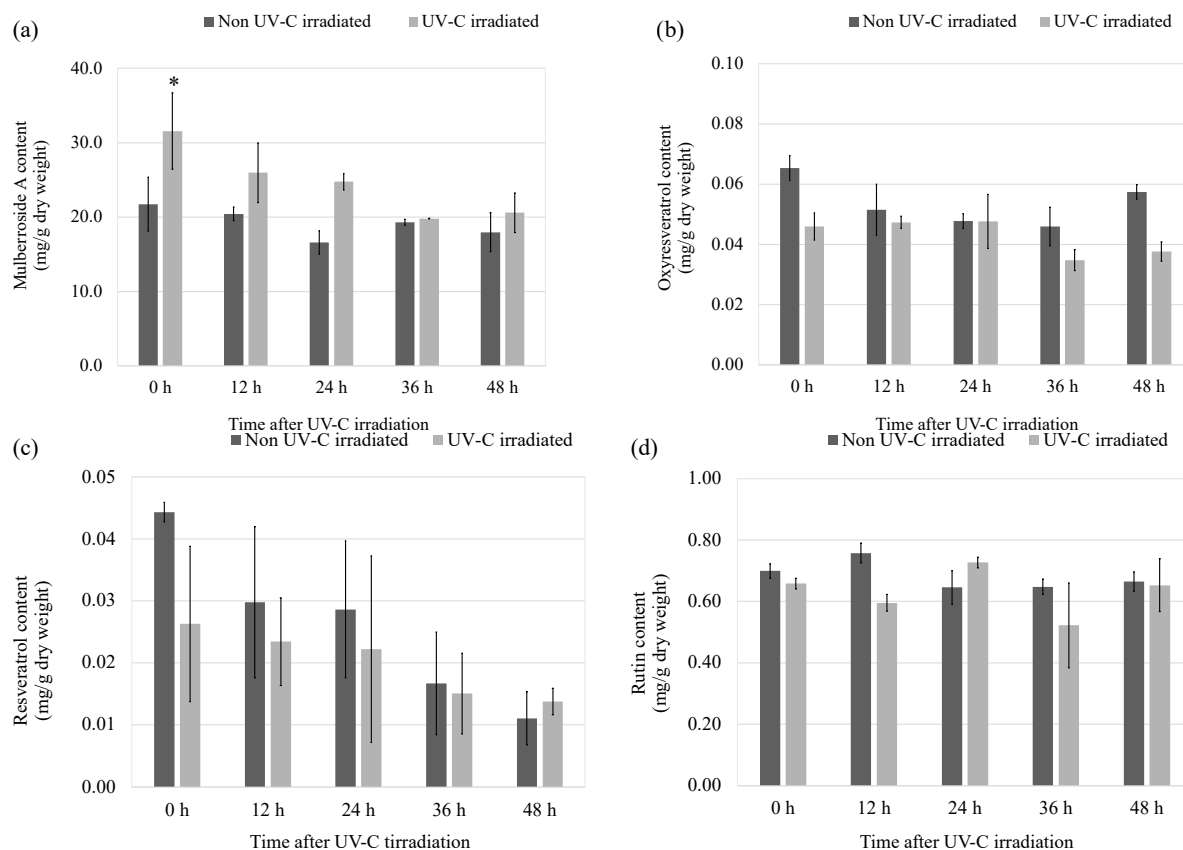
Cell suspension cultures of *M. alba* were treated with UV-C. As outlined in a previous study, biomass and mulberroside A production in cell suspension culture of *M. alba* were highest after 3–4 weeks of culture [16]. Therefore, UV-C treatment was performed on a 21-day-old cell suspension culture. The effect of time after UV-C treatment on phytochemical production was investigated (Fig. 2).

### Mulberroside A production

The effect of UV-C irradiation on mulberroside A production in cell suspension cultures of *M. alba* was studied. Based on the results of a previous study, mulberroside A could be released from cells into medium [16]. Therefore, both intracellular and extracellular mulberroside A contents were checked and reported as total mulberroside A content. Changes in total mulberroside A production in cell suspension cultures of *M. alba* occurred at different times after UV-C treatment (Fig. 2a). Total mulberroside A content in untreated cell suspension cultures ranged from  $16.60 \pm 1.58$  to  $21.75 \pm 3.62$  mg/g dry weight, while the value of  $19.81 \pm 0.04$  to  $31.58 \pm 5.14$  mg/g dry weight was found in treated cell suspension cultures. Overall, UV-C irradiation enhanced production of mulberroside A. A significantly higher total mulberroside A content was obtained from treated cells collected immediately after 20 min of UV-C irradiation. *Morus alba* cell suspension cultures irradiated with UV-C for 20 min and harvested immediately after UV-C treatment had total mulberroside A production 1.45 times higher than the untreated cells.

### Oxyresveratrol production

The content of oxyresveratrol (an aglycone form of mulberroside A) in cell suspension cultures of *M. alba* after UV-C irradiation was determined. Oxyresveratrol contents in untreated cell suspension cultures ranged from  $0.046 \pm 0.006$  to  $0.065 \pm 0.004$  mg/g dry weight (Fig. 2b). UV-C irradiation tended to decrease the production of oxyresveratrol in *M. alba* cell suspension cultures. However, no significant difference was observed between the UV-C treated cell suspension cultures and the controls.



**Fig. 2** Phytochemical contents in cell suspension cultures of *M. alba* treated with UV-C irradiation at different times after treatment; (a) mulberroside A contents, (b) oxyresveratrol contents, (c) resveratrol contents, and (d) rutin contents. \* indicates a significant difference compared to the control at the  $p$ -value  $< 0.01$

### Resveratrol production

The amounts of resveratrol in cell suspension cultures of *M. alba* tended to decrease over time after UV-C treatment (Fig. 2c). Resveratrol content in untreated cells was  $0.044 \pm 0.002$  mg/g dry weight and decreased to  $0.011 \pm 0.004$  mg/g dry weight after 48 h, while there was  $0.026 \pm 0.013$  mg/g dry weight in treated cells at 0 h, which decreased to  $0.014 \pm 0.002$  mg/g dry weight at 48 h after UV-C irradiation. No significant difference was observed between treated and untreated cells after UV-C irradiation.

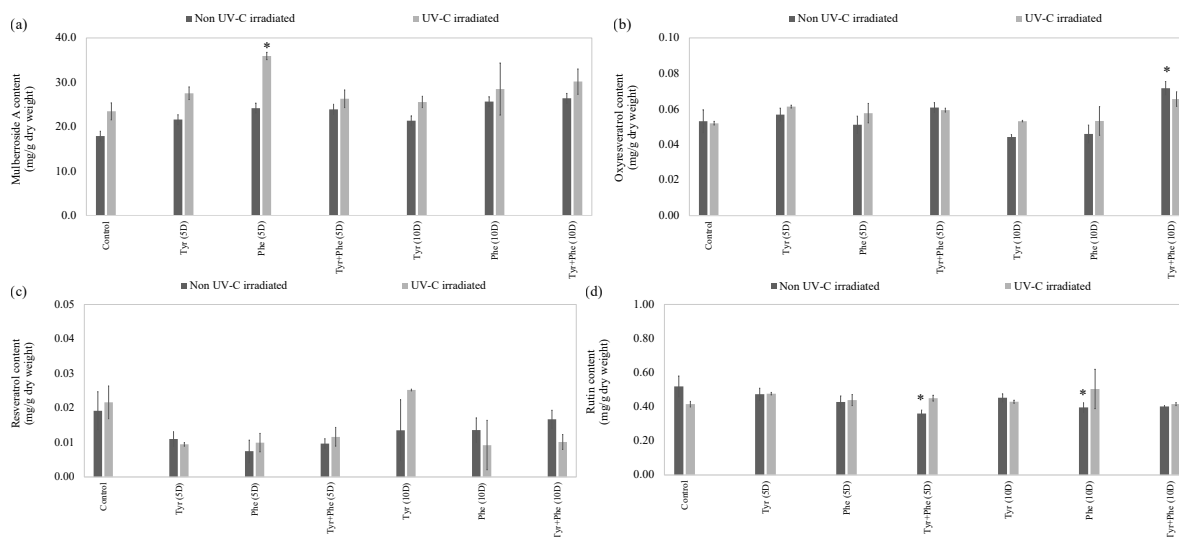
### Rutin production

Effect of UV-C irradiation on *M. alba* production of rutin, a flavonol compound in *M. alba*, was also investigated. Rutin contents in *M. alba* cell suspension cultures ranged from  $0.646 \pm 0.055$  to  $0.758 \pm 0.033$  mg/g dry weight and  $0.522 \pm 0.138$  to  $0.727 \pm 0.018$  mg/g dry weight in untreated

cells and treated cells, respectively (Fig. 2d). No significant difference in the amounts of rutin was observed.

### Effect of UV-C in combination with precursor feeding on phytochemical production in cell suspension cultures of *M. alba*

Based on the results of a previous experiment, UV-C irradiation significantly promoted the amount of mulberroside A, a major bioactive compound of *M. alba*, in cell suspension cultures when samples were harvested immediately after a 20-min UV-C treatment. Therefore, to study the effect of UV-C irradiation in combination with precursor feeding, the mentioned UV-C treatment conditions were used in this study. Phe and Tyr, either alone or in combination, were used as precursors. The amounts of total mulberroside A, oxyresveratrol, resveratrol, and rutin subjected to UV-C irradiation and precursor feeding are detailed in Fig. 3.



**Fig. 3** Phytochemical contents in cell suspension cultures of *M. alba* treated with L-phenylalanine and L-tyrosine, either single or in combination, followed by UV-C irradiation; (a) mulberroside A contents, (b) oxyresveratrol contents, (c) resveratrol contents, and (d) rutin contents. \* indicates a significant difference compared to the control at the  $p$ -value  $< 0.01$

### Mulberroside A production

The changes in mulberroside A production in cell suspension cultures of *M. alba* subjected to UV-C irradiation and precursor feeding are shown in Fig. 3a. Overall, either UV-C irradiation or precursor feeding could promote the production of mulberroside A. Significant differences in total mulberroside A from untreated, non-irradiated controls were observed in cell suspension cultures fed with Tyr for 5 d and irradiated with UV-C, fed with Phe for 5 d and irradiated with UV-C, fed with Phe for 10 d and irradiated with UV-C, and fed with both Tyr and Phe for 10 d and irradiated with UV-C. When compared with non-irradiated cells in the same treatment, significant differences were observed in cell suspension cultures fed with Phe for 5 d. UV-C irradiation together with precursor feeding enhanced total mulberroside A production in *M. alba* cell suspension cultures. The highest mulberroside A content was obtained from cell suspension cultures fed with Phe for 5 d and UV-C irradiation ( $35.93 \pm 0.81$  mg/g dry weight), which was doubled that obtained from non-irradiated and untreated cell suspension cultures ( $17.89 \pm 1.36$  mg/g dry weight).

### Oxyresveratrol production

The amounts of oxyresveratrol in cell suspension cultures of *M. alba* after precursor feeding and UV-C irradiation ranged from  $0.044 \pm 0.005$  to

$0.072 \pm 0.004$  mg/g dry weight (Fig. 3b). A significant difference from the untreated, non-irradiated control was observed in cell suspension cultures fed with both Phe and Tyr for 10 d without UV-C irradiation. Similar to a previous experiment, UV-C did not increase oxyresveratrol production in cell suspension cultures of *M. alba*.

### Resveratrol production

The contents of resveratrol in cell suspension cultures of *M. alba* ranged from  $0.007 \pm 0.003$  to  $0.025 \pm 0.001$  mg/g dry weight (Fig. 3c). No significant difference in the level of resveratrol was observed in all treatments.

### Rutin production

Similar to oxyresveratrol and resveratrol production in *M. alba* cell suspension cultures, precursor feeding and UV-C irradiation did not increase rutin content. The amounts of rutin in *M. alba* cell suspension cultures ranged from  $0.361 \pm 0.019$  to  $0.520 \pm 0.062$  mg/g dry weight (Fig. 3d). Significant decreases in rutin production were observed in non-irradiated cell suspensions cultures fed with Phe and Tyr for 5 d and non-irradiated cell suspensions cultures fed with Tyr for 10 d.

## DISCUSSION

UV-C irradiation has been commonly used to improve crop quality in grape berries. Several studies have reported the enhancement of stilbene and flavonoid production after UV-C irradiation. In *in vitro* cultures, UV-C promoted the accumulation of stilbenes in grape calli [20] and cell suspension cultures [14]. The stilbene biosynthetic pathway involves several enzymes, including phenylalanine ammonia lyase, cinnamic acid hydroxylase, caffeic acid 4-hydroxylase, styrene synthase, and stilbene synthase. A previous study reported that stilbene synthase (a key enzyme), and two other enzymes in stilbene biosynthesis were induced upon UV irradiation of leaves of various species of Vitaceae [21]. Therefore, the accumulation of stilbenes improved as a result of the increasing activities of these enzymes. In this study, UV-C irradiation increased the production of mulberroside A, a major bioactive stilbene, in cell suspension cultures of *M. alba*. The results concur with Wang et al [22], who reported significant enhancement of mulberroside A content in *M. alba* branches after UV-C irradiation. However, in the current study, UV-C irradiation did not significantly improve the production of oxyresveratrol and resveratrol in cell suspension cultures of *M. alba*. This could be because oxyresveratrol and resveratrol share the same biosynthetic pathway with mulberroside A, hence the biosynthesis was shifted to favor the production of mulberroside A.

UV-C acts as an abiotic elicitor that induces the short-time stresses and results in an enhancement of mulberroside A accumulation as a defense or stress-induced response. The elicitation of stilbene by UV-C is transient in several reported studies. In cell suspension cultures of *Vitis vinifera* L.cv. Cabernet Sauvignon, *trans*-resveratrol content increased gradually to the maximum at 48 h after UV-C irradiation, and then declined rapidly [14]. Similarly, the decrease of stilbene accumulation was observed after the maximum enhancement by UV-C irradiation in various grape varieties [23, 24]. Therefore, the harvest time after UV-C irradiation is a vital parameter for stilbene production. In this study, *M. alba* cell suspension cultures accumulated the highest content of mulberroside A when harvested immediately after UV-C irradiation, and production gradually decreased over time.

The addition of stilbene precursors, Phe and Tyr, either alone or in combination, increased production of mulberroside A in cell suspension cultures of *M. alba*. The highest mulberroside A content was

obtained from the *M. alba* cell suspension culture that was treated with 0.05 mM Phe 5 d before UV-C irradiation, which was double the mulberroside A content in the non-irradiated and untreated cells. The content of mulberroside A produced in these conditions was higher than that reported from intact leaves, roots, stems, and barks of *M. alba* [19, 25]. Similarly, Inyai et al [11] reported increased mulberroside A production from immobilized cell cultures of *M. alba* fed with Phe and Tyr. The current study concurs with a previous report which showed that <sup>13</sup>C-labeled Phe and Tyr precursors were both incorporated into the cinnamoyl moiety in the biosynthetic pathway of mulberroside A [26].

## CONCLUSION

Several studies have reported the effect of UV-C irradiation on stilbene production in plants. In this study, the effects of precursor feeding in combination with UV-C irradiation on phytochemical production in cell suspension cultures of *M. alba* are reported for the first time. The combined UV-C irradiation and precursor feeding doubled the production of mulberroside A (a major bioactive stilbene) in *M. alba* L. cell suspension cultures. The content of mulberroside A produced in these conditions was higher than that reported from the intact leaves, roots, stems, and bark of *M. alba* [18, 21]. Therefore, the use of precursor feeding in combination with UV-C irradiation could be an alternative method for the production of mulberroside A in *M. alba*.

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