Induced extracellular production of viniferins in grapevine cell culture medium by elicitation with methyl jasmonate and stevioside

Yu Jeong Jeong
Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeongeup 56212, Republic of Korea

Su Hyun Park
Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeongeup 56212, Republic of Korea. Department of Plant Biotechnology, College of Agriculture and Life Science, Chonnam National University, Gwangju 61186, Republi

Sung-Chul Park
Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeongeup 56212, Republic of Korea

Soyoung Kim
Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeongeup 56212, Republic of Korea. Department of Plant Biotechnology, College of Agriculture and Life Science, Chonnam National University, Gwangju 61186, Republi

Tae Hee Kim
Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeongeup 56212, Republic of Korea

Jiyoung Lee
Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeongeup 56212, Republic of Korea

Suk Weon Kim
Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeongeup 56212, Republic of Korea

Young Bae Ryu
Functional Biomaterials Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeongeup 56212, Republic of Korea

Jae Cheol Jeong
Biological Resource Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Jeongeup 56212, Republic of Korea

Cha Young Kim (kimcy@kribb.re.kr)
Korea Research Institute of Bioscience and Biotechnology https://orcid.org/0000-0001-7372-186X

Research

Keywords: Grapevine, Cell cultures, Stilbenes, Viniferins, Resveratrol, Stevioside

Posted Date: March 18th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-17669/v1

Version of Record: A version of this preprint was published on July 14th, 2020. See the published version at https://doi.org/10.1186/s40643-020-00329-3.

Abstract

We report the high production of stilbenes, including resveratrol and viniferins, in grapevine (*Vitis labruscana* L.) cell cultures through elicitation with methyl jasmonate (MJ) and stevioside (STE). Methyl-β-cyclodextrin (CD-M) is widely used as a solubilizer for resveratrol production. For the first time, we used STE as a solubilizer for stilbene production in plant cell cultures. MJ was most effective in activating *VvSTS* expression and stimulating stilbene biosynthesis and induced accumulation of *trans*-piceid in the cells. High levels of *trans*-resveratrol and δ-viniferin production were observed in the culture medium when CD-M and STE, respectively, were used as solubilizers, but neither were significantly elevated within the cells. Maximum production of *trans*-resveratrol (12.2 mg/L) and δ-viniferin (892.2 mg/L) was observed 5 days after elicitation of cells with MJ and STE in shake flask cultures. Notably, predominant production of δ-viniferin and *trans*-resveratrol was observed in shake and static flask culture medium, respectively. Furthermore, stilbene compounds of resveratrol, e-viniferin, and δ-viniferin were mainly produced in a 3 L bioreactor culture following elicitation of cells with MJ and STE. These results provide new strategies for conditional, high-level production of resveratrol and viniferins in cell cultures by utilizing the solubilizing properties of STE or CD-M.

Introduction

Plants synthesize a wide range of secondary metabolites in response to various environmental stresses (Langcake and Pryce 1977; Zamboni et al. 2006). Stilbenes, particularly resveratrol and viniferins, have attracted extensive attention and interest due to their health benefits. Grapevines produce stilbenes derived from the phenylpropanoid pathway. Plant stilbenes are phytoalexins that accumulate in a small number of plant species in response to biotic and abiotic stresses and are mainly derivatives of the monomeric unit *trans*-resveratrol (*trans*-3,5,4'-trihydroxystilbene) (Donnez et al. 2011; Jeandet et al. 2002; Jeandet et al. 2020; Sotheeswaran and Pasupathy 1993). Resveratrol is a naturally occurring stilbene produced in more than 70 plant species, including grapevines, peanuts, and Japanese knotweed (Aggarwal et al. 2004; Bhat and Pezzuto 2002; Jang et al. 1997; Jeong et al. 2016). The biosynthesis of resveratrol is controlled by stilbene synthase (STS). UV-irradiation induces accumulation of stilbenes by inducing *STS* gene expression in grape berries (Pan et al. 2009; Pezet et al. 2003). Methyl jasmonate (MJ) also induces accumulation of resveratrol and *e*-viniferin, a resveratrol dehydrodimer in grapevines (Vezzulli et al. 2007). In addition, pathogen infection stimulates biosynthesis of *d*- and *e*-viniferin in grapevine leaves (Bavaresco et al. 1997; Pezet et al. 2003).

Resveratrol is the skeleton for producing various derivatives. Piceid and pinostilbene or pterostilbene are produced through glycosylation and methylation of resveratrol by UDP-glycosyltransferases (UGT) and resveratrol *O*-methyl transferases (ROMT), respectively, while oxidative dimerization of two resveratrol units by (4-hydroxystilbene) peroxidases leads to production of *d*- or *e*-viniferin (Jeandet et al. 2002; Jeong et al. 2014; Jeong et al. 2015; Xue et al. 2014). Resveratrol has been implicated in a large number of beneficial effects on human health (Bradamante et al. 2004; Goswami and Das 2009; Valenzano et al.

2006). Due to its therapeutic value, it is in demand for nutraceutical, cosmetic, and pharmaceutical applications.

Resveratrol and its derivatives are currently produced by extraction from plant materials, chemical synthesis, and bio-production (Donnez et al. 2009; Jeandet et al. 2016). Two major biotechnological approaches are currently widely applied in bio-production: (i) the use of genetically-engineered microorganisms or plants (Donnez et al. 2009; Donnez et al. 2011; Jeandet et al. 2018; Wang and Yu 2012; Wu et al. 2013) and (ii) the use of *in vitro* plant cell cultures under controlled conditions (Chastang et al. 2018; Donnez et al. 2009; Donnez et al. 2011; Jeandet et al. 2016), especially grapevine cell cultures. The bio-production of resveratrol and its derivatives in grapevine cell cultures has been reported as a promising biotechnological alternative to their plant extraction and chemical synthesis. Plant cell cultures have been used extensively for bio-production of valuable secondary metabolites under controlled conditions independent of climatic changes or soil conditions (Giri and Zaheer 2016; Hussain et al. 2012; Jeandet et al. 2016; Jeong et al. 2018; Mulabagal and Tsay 2004; Murthy et al. 2014). To date, elicitation with MJ combined with dimethyl-b-cyclodextrins (CD-M) has proven the most efficient approach for high production of resveratrol and its derivatives in grapevine cell cultures (Bru et al. 2006; Lijavetzky et al. 2008; Martinez-Esteso et al. 2009; Martínez-Márquez et al. 2016) (Donnez et al. 2009; Lambert et al. 2019). Furthermore, it has been shown that stevioside (STE), a diterpene glycoside comprising an aglycone (steviol) and three molecules of glucose with mono- and disaccharide carbohydrate residues at the C13 and C19 positions, possesses solubilizing properties (Liu 2011; Uchiyama et al. 2012; Wan et al. 2013).

The aim of this study was to investigate the effects of solubilizing agents such as CD-M and STE in combination with MJ as an elicitor on the bio-production of resveratrol and viniferins in grapevine cell cultures. Herein, we show that the combined applications of MJ and CD-M or STE offer improved and preferential production of resveratrol and viniferins in cell culture media. This is the first study showing that STE can be used as a solubilizer in combination with MJ to trigger increased production of viniferins in grapevine cell culture medium. Moreover, we suggest that the use of CD-M and STE with solubilizing properties in plant cell cultures makes culture systems more reliable and productive for induced biosynthesis and secretion of secondary metabolites.

Materials And Methods

Plant material

Grapevine (*Vitis labruscana* L. cv. Campbell Early) was obtained from a commercial vineyard in Korea for this study. Under aseptic conditions, the anther explants were surface-sterilized with 70% (v/v) ethanol for 30 s, subsequently soaked in a 20% (v/v) solution of commercial sodium hypochlorite (NaClO) containing 4% (w/v) active ingredient for 15–20 min, and rinsed three times with sterile distilled water before being dried on sterile filter paper.

Callus induction and cell suspension cultures

The calli were induced from the anther explants of grapevines on MS1D (Murashige and Skoog basal salt mixture supplemented with 1.0 mg/L 2,4-D and 3 % (w/v) sucrose) medium containing 4 g/L Gelrite (Duchefa Biochemie B.V., Haarlem, The Netherlands) in darkness at 24°C. The homogenous calli selected from callus cultures were maintained by transferring the relatively friable portion of a callus onto MS1D solid medium every 4 weeks. Finally, the best calli were deposited at the Korean Collection for Type Cultures (KCTC; http://bioproduct.kribb.re.kr) as bio-product BP1347372. Grapevine cell suspension cultures were initiated and established by inoculating small pieces of homogeneous and friable calli in MS1D liquid medium as described by Jeong *et al.* (2018). The cell cultures were agitated at 90 rpm on a rotary shaker and maintained by sub-culturing every 4 weeks in MS1D liquid medium in darkness at 24°C. The cultured cells were harvested via filtration through a nylon mesh filter (100 μm), freeze-dried, and weighed to determine grams of dry weight (DW).

Cell suspension growth curve

A cell growth curve was used to determine the most suitable period for harvesting. Cell growth was measured based on dry weight of cultures. For this experiment, approximately 2.5 g fresh weight (FW) of the established cells (i.e., 7 days old), were transferred into 25 mL of fresh medium in 125 mL Erlenmeyer flasks. To determine cell density at each time point, the harvested cells were collected and dried at 60°C. The dry weight of the cultured cells was determined after drying the harvested cells. The cell suspension cultures were monitored at 2-day intervals for 16 days. Measurements were repeated three times.

Elicitation of grapevine cell suspension cultures

Cell suspension cultures were sub-cultured into 100 mL MS1D liquid medium in 500 mL Erlenmeyer flasks and placed on a rotary shaker (90 rpm) at 24°C under dark conditions for 7 days as described by Jeong *et al*. (2018). For RT-PCR analysis, pre-cultured cells (6 mL aliquots) were transferred into each well of a 6-well tissue plate. Twelve hours later, the cells were treated for 48 h with various elicitors, such as methyl jasmonate (MJ), salicylic acid (SA), abscisic acid (ABA), ethephon (ET), flagellin 22 peptide (Flg22), methyl viologen (MV), and chitosan (Chi). For stilbene production in 500 mL flask cultures, cells (approximately 10 g) pre-cultured in 100 mL MS1D liquid medium for 7 days were treated with 100 μM MJ and/or 50 mM stevioside (Daepyung, Ltd., Seongnam, Korea) or 50 mM methyl-b-cyclodextrin (Sigma-Aldrich, St. Louis, MO, USA) for 5 days. In bioreactor cultures, cells (approximately 10 g/100 mL, 3 flasks) pre-cultured for 14 days were transferred into a 3 L bioreactor containing 1.2 L MS1D medium. After cultivation for 5 days, the cells were treated for 5 days with 100 μM MJ and/or 50 mM stevioside. The elicited samples (cells and medium) were harvested by filtration through a nylon mesh filter (100 μm). The elicitation experiments were carried out with three replicates.

Monitoring of cell viability

Cell viability was monitored by staining cell suspensions with 0.1% Evans blue (Sigma-Aldrich) as previously described by Jeong *et al.* (2018).

RNA extraction and reverse transcription (RT)-PCR analysis

Total RNA isolation, cDNA synthesis, and RT-PCR were performed as previously described (An et al. 2015; Jeong et al. 2018). The beta-actin (*VvActin7*, XM_002282480.4) gene in grapevines was used as an internal control in RT-PCR. The primers used in this study are as follows: *VvSTS*-F, 5'-ATGGCTTCAGTTGAGGAAATCAGA-3'; *VvSTS*-R, 5'-TTAATTTGTCACCATAGGAATGCTA-3'; *VvActin7*-F, 5'-TGCTGACAGAATGAGCAAGG-3'; *VvActin7*-R, 5'-TACTAAGAAGCTTTCAACCCAGTATA-3'

Resveratrol and viniferin extraction and HPLC analysis

For analysis of resveratrol and viniferin contents in the callus cells of grapevine cell cultures, freeze-dried calli (approximately 1 g) were extracted with 5 mL of 80% methanol. After extraction, samples were concentrated by evaporation and dissolved in 0.5 mL 80% methanol. For analysis of stilbenes produced in cell culture medium, 1 mL of the culture medium was extracted with an equal volume of ethyl acetate, and samples were concentrated by evaporation and dissolved in 1 mL 80% methanol. The extract samples were purified using a 0.2 μm hydrophilic PTFE membrane filter (Advantec MFS, Inc., Dublin, CA, USA) before HPLC analysis. The concentration of *trans*-resveratrol and e-viniferin produced by grapevine cell cultures was calculated by comparison with the known concentrations (mg/L) of *trans*-resveratrol and e-viniferin (Sigma-Aldrich) as standards. The d-viniferin compound was isolated and purified from the grapevine cell cultures for use as a standard. Concentration is shown at the milligrams per liter scale (mg/L). d-Viniferin was identified by NMR analysis (Fig. S4).

HPLC and LC-MS analyses were performed using an Agilent 1200 system (Agilent Technologies, Santa Clara, CA, USA) as previously described by Jeong *et al.* (2018). Each stilbene compound was identified by comparison with commercial standards. The quantity of stilbene compounds in each sample was determined from the standard curve.

Results

Establishment and optimization of grapevine cell cultures

The grapevine cell suspension cultures were established using calli derived from anthers and maintained by sub-culturing homogeneous cells in MS1D liquid medium several times at 2-week intervals. As shown in Fig. 1, grapevine cell growth exhibited a similar growth pattern as that observed in other plant cell suspension cultures (Muatafa et al. 2011). An initial lag phase was observed during the first 1–4 days, followed by an exponential rise in cell growth until day 12, with maximum cell density on day 12 (approximately 17.3 g DW). During the stationary phase (12–16 days), cell density declined after day 12 along with a gradual decrease in cell viability. This observation indicated that 4–8 days of culture in the early exponential phase was likely the best time to harvest cells for subsequent experiments.

Expression analysis of stilbene synthase gene following elicitor application

Resveratrol biosynthesis is controlled by stilbene synthase (STS), which controls the entry point into the stilbene biosynthetic pathway (Fig. 2A). Thus, to obtain the best conditions for resveratrol production in grapevine cell suspension cultures, the expression patterns of the grapevine stilbene synthesis gene (*VvSTS*) were analyzed by RT-PCR using flask culture samples treated with stress hormones as elicitors (Fig. 2B; Fig. S1). As shown in Fig. 2B, *VvSTS* expression increased greatly 3 h after treatment with MJ, SA, and ABA. *VvSTS* expression levels in MJ-treated cells were significantly higher than those in SA- or ABA-treated cells. Ethephon also positively affected *VvSTS* expression, with a maximum level at 12 h. However, *VvSTS* did not respond to the application of Flg22, MV, or chitosan (Fig. S1). Higher levels of *VvSTS* expression were observed in cell suspension cultures elicited with 100 μM MJ than in those elicited with 300 μM MJ (Fig. S1). In this study, we thus used 100 μM MJ as an elicitor for the induction of resveratrol production in the grapevine cell cultures.

Enhanced production of resveratrol and viniferin in grapevine cell cultures

We further examined whether up-regulation of *VvSTS* by MJ application induces enhanced production of stilbenes, such as resveratrol and piceid. Resveratrol and piceid production has been reported in grapevine cell cultures upon stimulation of cells by several elicitors, including MJ (Almagro et al. 2014; Belchí-Navarro et al. 2012; Belhadj et al. 2008; Donnez et al. 2011; Lijavetzky et al. 2008; Martínez-Márquez et al. 2016). Thus, endogenous accumulation within the cells and release into the culture medium of resveratrol and its derivatives were measured in grapevine cell cultures treated with 100 μM MJ. Fig. 3 shows that the accumulation of *trans*-piceid, a glycosylated form of *trans*-resveratrol, considerably increased within the cells when MJ was added to grapevine cell cultures. However, no significant increase in *trans*-resveratrol or *trans*-piceid contents was detected after treatment with ABA or SA. The maximum *trans*-piceid production of 54.647 μg/g DW was observed 48 h after treatment with 100 μM MJ. We noticed that *trans*-piceid production was detected in the cells but not in the culture medium (data not shown).

Previous studies (Lijavetzky et al. 2008; Liu 2011; Wan et al. 2013) reported that cyclodextrin and stevioside effectively enhance the solubility of water-insoluble resveratrol. Thus, we investigated whether *trans*-resveratrol or *trans*-piceid production can be improved by the use of solubilizing agents, such as CD-M and STE, in the cell cultures. As shown in Fig. 4 and Fig. S3, *trans*-resveratrol production was considerably increased by addition of both MJ and CD-M in the culture medium, but not in the cells, reaching 371.9 mg/L 5 days after elicitation in shake flask cultures. However, as shown in Fig. 3, *trans*-piceid production in the cells elicited by MJ was not detected in both the cells and culture medium. Interestingly, co-treatment with MJ and STE greatly induced high-level production of d-viniferin as a major compound along with a small amount of resveratrol in the culture medium, but not in the cells. The maximum production of δ-viniferin (892.2 mg/L) in the extracellular medium was observed on day 5 following elicitation of the cells with MJ and STE in shake flask cultures. Unlike application of MJ and/or CD-M, we observed that the cell cultures gradually turned brown following co-treatment with MJ and STE, indicating the accumulation of viniferin (Fig. 4A). These results suggest that CD-M and STE preferentially allow release of *trans*-resveratrol and d-viniferin, respectively, into the culture medium.

Since d-viniferin production in culture medium was greatly enhanced by STE application, we examined whether the solubilizing effect of STE on water-insoluble resveratrol and viniferin is concentration-dependent. As shown in Fig. 4C and 4D, STE effectively induced solubilization of hydrophobic *trans*-resveratrol and d-viniferin in water solutions. The solubilities of *trans*-resveratrol and d-viniferin in water without STE were 92.0 µg/mL and 419.7 µg/mL, respectively. As STE concentration increased to approximately 2.5% (w/v), higher solubility was observed for d-viniferin than for *trans*-resveratrol. The solubility of d-viniferin was enhanced at even low concentrations (0.1–0.6%) of STE (Fig. 4D). In the presence of 1% STE (w/v), *trans*-resveratrol and d-viniferin were solubilized in water to about 309.0 µg/mL and 897.8 µg/ml, respectively. Most *trans*-resveratrol and d-viniferin (more than 90%) were solubilized in approximately 3% and 1% STE (w/v) solutions, respectively. These results suggest that STE effectively enhances the water solubility of water-insoluble *trans*-resveratrol and d-viniferin.

Conditional production of *trans*-resveratrol and d-viniferin in grapevine cell cultures

As shown in Fig. 4, the combined use of MJ and STE predominantly produced δ-viniferin along with a trace amount of resveratrol in shake flask culture medium. Interestingly, we found that δ-viniferin production was greatly reduced while *trans*-resveratrol accumulation was highly enhanced under stationary flask culture (Fig. 5; Fig. S3). Furthermore, we noticed that a mixture of stilbenes, including *trans*-resveratrol, e-viniferin, and δ-viniferin were produced in a 3 L bioreactor culture 5 days following elicitation of cells with MJ and STE (Fig. 5). These results suggest that stilbene production (resveratrol or viniferin) is influenced by cultivation conditions in grapevine cell cultures. In other words, it is possible to obtain specific compounds by controlling cultivation conditions (with or without shaking) for the preferential production of resveratrol or viniferin in flask cultures.

As shown in Fig. 4A and 5A, the cultured cells became a darker brown color 5 days post-elicitation with MJ and STE, unlike untreated control cells or cells elicited with MJ and CD-M, indicating the accumulation of oxidation products. Cell growth was also observed to be somewhat reduced by the application of MJ and STE. This application probably caused some cell death, but the brown color may have been the result of accumulation of viniferin, oxidized dimers of resveratrol, produced during cell culture. In general, resveratrol and viniferin appear white and brown, respectively. As shown in Fig. S2, there were no significant differences among numbers of blue-stained cells observed after treatment with MJ alone or MJ and STE or CD-M compared to that in untreated control cells, indicating that the concentrations of MJ and STE or CD-M used in this study were not cytotoxic.

Discussion

In recent years, resveratrol has attracted considerable attention as a star polyphenol compound due to its abundant health benefits. Resveratrol oligomers also have numerous impressive bioactivities, but few studies have been performed due to their trace amounts in natural resources (Shen et al. 2009). Thus, there have been many efforts to enhance production of resveratrol and its derivatives in plants and microorganisms through metabolic engineering (Beekwilder et al. 2006; Delaunois et al. 2009; Donnez et

al. 2009; Jeong et al. 2014; Jeong et al. 2015; Jeong et al. 2016; Nivelle et al. 2017; Shin et al. 2011; Yu et al. 2006). Currently, plant cell suspension cultures are a promising approach for resveratrol production under controlled aseptic conditions. This biotechnological production is commonly coupled with cell culture-based biotransformation using an exogenous supply of biosynthetic precursors or various elicitors.

Elicitation of plant cells has been shown to be the most efficient way to induce and boost the biosynthesis of useful secondary metabolites (Namdeo 2007; Zhao et al. 2005). To date, there have been many reports that MJ application can effectively promote resveratrol production in grapevine cell suspension cultures (Almagro et al. 2014; Belchí-Navarro et al. 2012; Belhadj et al. 2008; Donnez et al. 2009; Lambert et al. 2019; Lijavetzky et al. 2008; Martínez-Márquez et al. 2016; Tassoni et al. 2005). Furthermore, Tassoni *et al.* (2005) reported that MJ effectively stimulated endogenous accumulation of resveratrol and its release into the culture medium. In this study, we established an efficient method for producing *trans*-resveratrol and d-viniferin in culture medium (i.e., extracellular compartment) of grapevine cell cultures using CD-M and STE as solubilizers. Upon MJ elicitation, CD-M and STE were highly effective in promoting accumulation of *trans*-resveratrol and d-viniferin in the culture medium, respectively (Fig. 4). However, there was no accumulation or release of *trans*-resveratrol or d-viniferin into the culture medium in the absence of CD-M or STE. Our data represent the first report on the extracellular production of d-viniferin in culture medium of MJ-treated cells using STE as a solubilizer. Additionally, the use of CD-M allowed the cells to release *trans*-resveratrol into the culture medium in response to MJ application (Fig. 4). Likely, CD-M is thus able to encapsulate *trans*-resveratrol but it is also considered as an elicitor. Unlike CD-M, however, STE alone was hardly able to induce the *VvSTS* expression and resveratrol biosynthesis as an elicitor (data not shown). Previous studies have demonstrated that cyclodextrins promote the accumulation and release of resveratrol in plant cell cultures (Bru et al. 2006; Donnez et al. 2009; Lambert et al. 2019; Lijavetzky et al. 2008; Morales et al. 1998). We are also the first to report that STE facilitates production and secretion of d-viniferin into the culture medium. Until now, there has been no experimental evidence showing how STE promotes secretion of *trans*-resveratrol and d-viniferin to the extracellular medium in grapevine cells or any other plant cell systems. We speculate that STE enabled secretion of *trans*-resveratrol into the extracellular medium (i.e., apoplastic space) via uncharacterized membrane transporters in response to MJ elicitation. After being transported out of cells, the resveratrol monomer might undergo oxidative dimerization to form resveratrol dimers of viniferin due to oxidizing conditions. Several types of membrane transporters, including ATP-binding cassette (ABC) transporters, are known to be involved in the secondary metabolite transport process (Grotewold 2004; Yazaki 2005). However, further investigation will be needed to gain insight into the transport of *trans*-resveratrol and d-viniferin out of the cells in grapevine cell cultures. Unlike cyclodextrins, the STE-resveratrol complex likely favors itself with access to extracellular oxidizing enzymes (e.g., peroxidases and polyphenol oxidases) present in the extracellular compartment (i.e., apoplastic space), thereby leading to the formation of viniferins in the culture medium. Indeed, Calderon *et al*. (1994) previously reported that a cell wall-bound peroxidase catalyzes dimerization of resveratrol to form viniferin in grapevine cell suspension cultures. In addition, Wan *et al*. (2013) demonstrated that STE effectively

improves the water solubility of resveratrol by incorporation of a water-soluble STE-resveratrol complex. In the present study (Fig. 4; Fig. S3), we also showed that STE has a similar solubilizing effect on water-insoluble viniferin as well as resveratrol. It has been shown that STE has characteristic properties of micelle-like nanostructures that allow it to self-assemble into micelles in aqueous solutions (Liu 2011; Uchiyama et al. 2012; Wan et al. 2013; Zhang et al. 2011). These characteristics of the STE structure likely include the combination of hydrophobic diterpene and hydrophilic sugar side chains. It was hypothesized that there is an interaction between the hydrophobic core of STE and resveratrol in STE self-assembled micelles that enhances the solubility of resveratrol. In our study, for the first time, we applied the biosurfactant STE as a solubilizer to enhance the extracellular production of resveratrol in a plant cell culture system. Thus, it would be very interesting to investigate whether STE similarly enhances the solubility of other polyphenolic compounds, such as curcumin and paclitaxel, with poor water solubility in plant cell cultures. We also observed d-viniferin production in flask cultures supplied with oxygen by shaking, whereas *trans*-resveratrol was produced in stationary cultures without agitation after the cells were treated with MJ and STE (Fig. 5). As described above, these findings suggest that STE encapsulates the resveratrol synthesized in the elicited cells (within STE micelles), which then secrete resveratrol complexes into the extracellular compartment, where resveratrol might be converted to viniferin by oxidative dimerization.

Interestingly, we also noticed that d-viniferin was mainly produced in shake flask cultures upon elicitation with MJ and STE, while e-viniferin was produced in a 3 L bioreactor culture in addition to *trans*-resveratrol and d-viniferin. It seems that the grapevine cell cultures in the bioreactor might synthesize the e- and d-viniferin in addition to *trans*-resveratrol because the bioreactor culture undergoes more shear stress resulting from agitation and aeration compared to the flask culture. Donnez *et al*. (2011) demonstrated that grapevine cell cultures (41B cells) are very sensitive to agitation speed and aeration. They also observed production of e- and d-viniferin as well as resveratrol in the cell extract of MJ-elicited cell cultures in a 2 L bioreactor. However, they did not determine whether these compounds were produced in the culture medium or in the cells. In the present study (Fig. 5), the high production level of *trans*-resveratrol, e-viniferin, and d-viniferin in a 3 L bioreactor was detected in the culture medium upon elicitation with MJ and STE, suggesting that they were secreted from cells in high quantities. As suggested above, however, the mechanism of viniferin biosynthesis by certain peroxidases remains to be elucidated.

By further optimization of plant cell culture conditions and metabolic pathway engineering strategies, production of resveratrol and viniferin could be further enhanced. For example, it would be quite interesting to examine whether the addition of resveratrol precursors, such as phenylalanine or *p*-coumaric acid, to the culture medium would increase the productivity of grapevine cell cultures. In the future, scaling of grapevine cell cultures to the industrial level will need to be achieved and optimized to produce large quantities of *trans*-resveratrol as well as e- and d-viniferin using MJ and STE.

Conclusions

For the first time, we used STE as a solubilizer for the bio-production of secondary metabolites in plant cell cultures. The use of MJ in combination with CD-M or STE represents an effective elicitation strategy for enhancing the production of *trans*-resveratrol and d-viniferin, respectively, in grapevine cell cultures, as well as their high production and secretion outside cells in the culture medium. In particular, the solubilizer STE could be applied for enhanced production and secretion of other water-insoluble metabolites/substances in plant cell cultures.

Abbreviations

MJ: methyl jasmonate; STE: stevioside; CD-M: methyl-β-cyclodextrin; UGT: UDP-glycosyltransferase; ROMT: resveratrol *O*-methyl transferase; DW: dry weight; FW: Fresh weight; SA: salicylic acid; ABA: abscisic acid; ET: ethephon; Flg22: flagellin 22 peptide: MV: methyl viologen; Chi: chitosan; RT-PCR; reverse transcription-polymerase chain reaction; HPLC: high performance liquid chromatography; STS: stilbene synthase

Declarations

Acknowledgements

We thank the members of the Korean Collection for Type Cultures (KCTC) at KRIBB for valuable supports of plant cell lines.

Funding

This work was supported by grants from the KRIBB Initiative Program and the Next-Generation BioGreen 21 Program (SSAC, Grant #: PJ01318604), Rural Development Administration, Republic of Korea.

Author's contributions

C.Y.K. and J.C.J. conceived and supervised this study; C.Y.K., J.C.J., and Y.J.J. designed the experiments; Y.J.J., S.H.P., T.H.K., and S.W.K. performed the grapevine cell cultures; Y.J.J. and S.C.P. performed the RT-PCR experiments; Y.J.J., S.H.P., and J.L. performed the Evans blue staining and water solubility experiments; Y.B.R., S.K., and S.H.P. performed the HPLC, LC-MS, and NMR experiments. C.Y.K., J.C.J., and Y.J.J. wrote the manuscript with assistance from the other authors.

Availability of data and materials

All data generated or analyzed during this study are included in this article and its supplementary information files.

Ethics approval and consent to participate

All authors have read and agreed the ethics for publishing the manuscript.

Consent for publication

All authors approved the consent for publishing the manuscript to Bioresources and Bioprocessing.

Competing Interests

The authors declare no conflict of interest.

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Figures

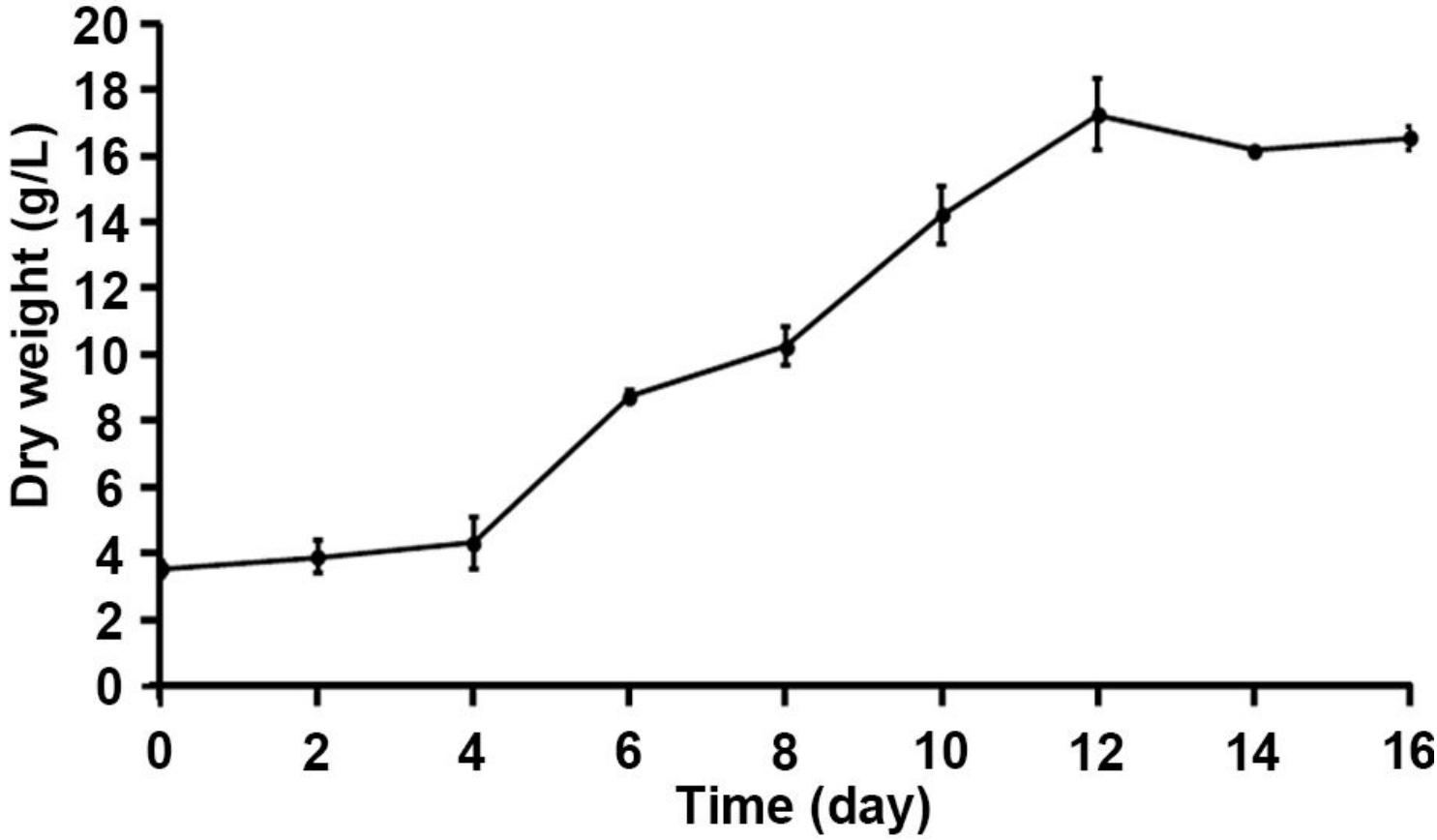

Figure 1

The growth curve measurement in grapevine cell cultures. Growth of cells in flask culture was measured at different time points. Three replications were performed for each sample. The maximum biomass growth was achieved on day 12 of grapevine cell cultures.

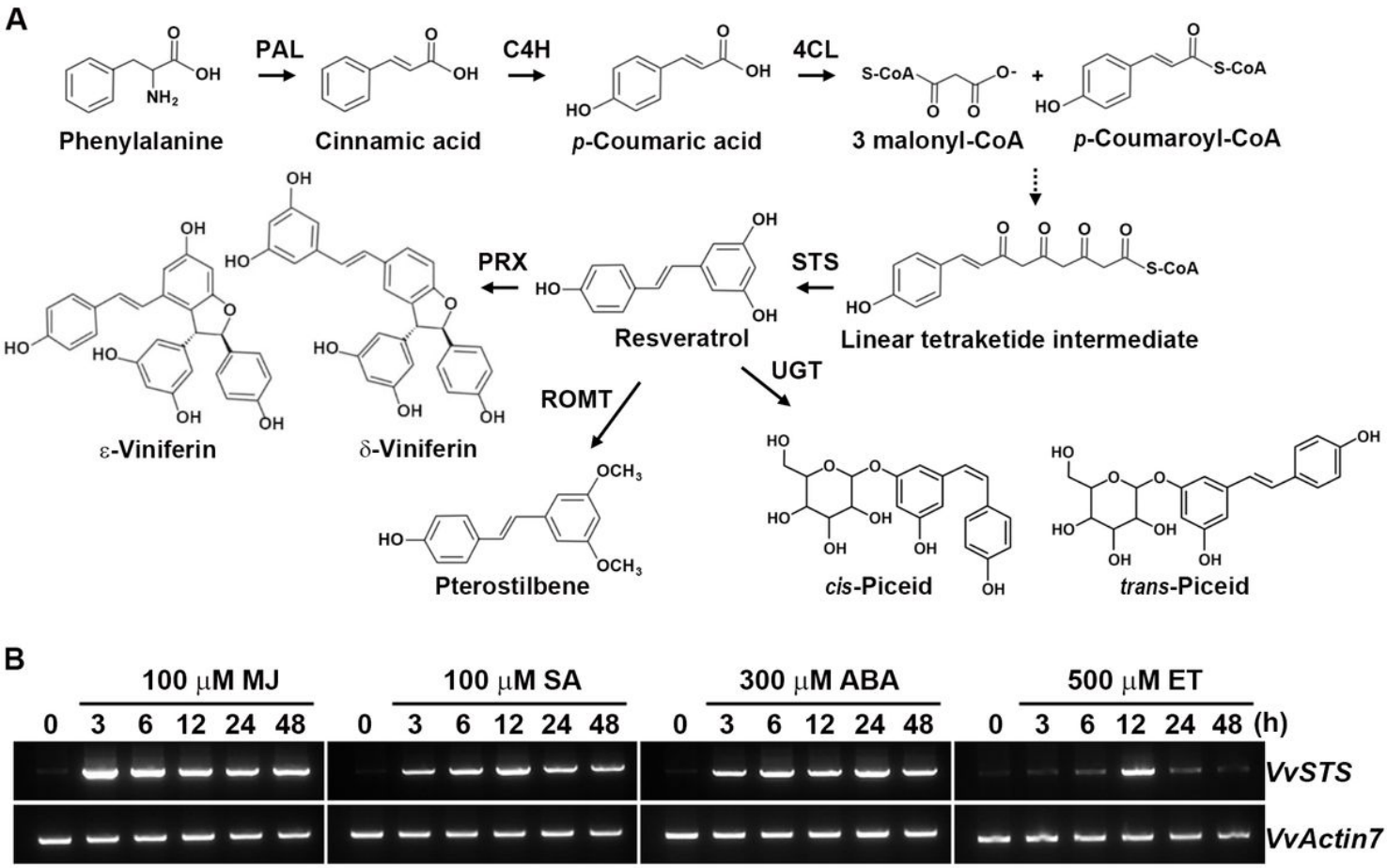

Figure 2

(A) Proposed stilbene biosynthetic pathway in grapevines and (B) effects of various elicitors on stilbene synthase gene (VvSTS) expression in grapevine cell cultures. The cells were pre-cultured for 7 days and harvested at different time points after elicitation for RT-PCR analysis. The elicitors used are as follows: MJ, methyl jasmonate; SA, salicylic acid; ABA, abscisic acid; ET, ethephon. VvActin7 (XM_002282480.4) was used as a quantitative control. The sequential actions of PAL, C4H, 4CL, STS, and UGT or PRX result in the conversion of phenylalanine to stilbenes. PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumarate-CoA-ligase; STS, stilbene synthase; UGT, UDP-glucosyltransferase; ROMT, resveratrol O-methyltransferase; PRX, peroxidase.

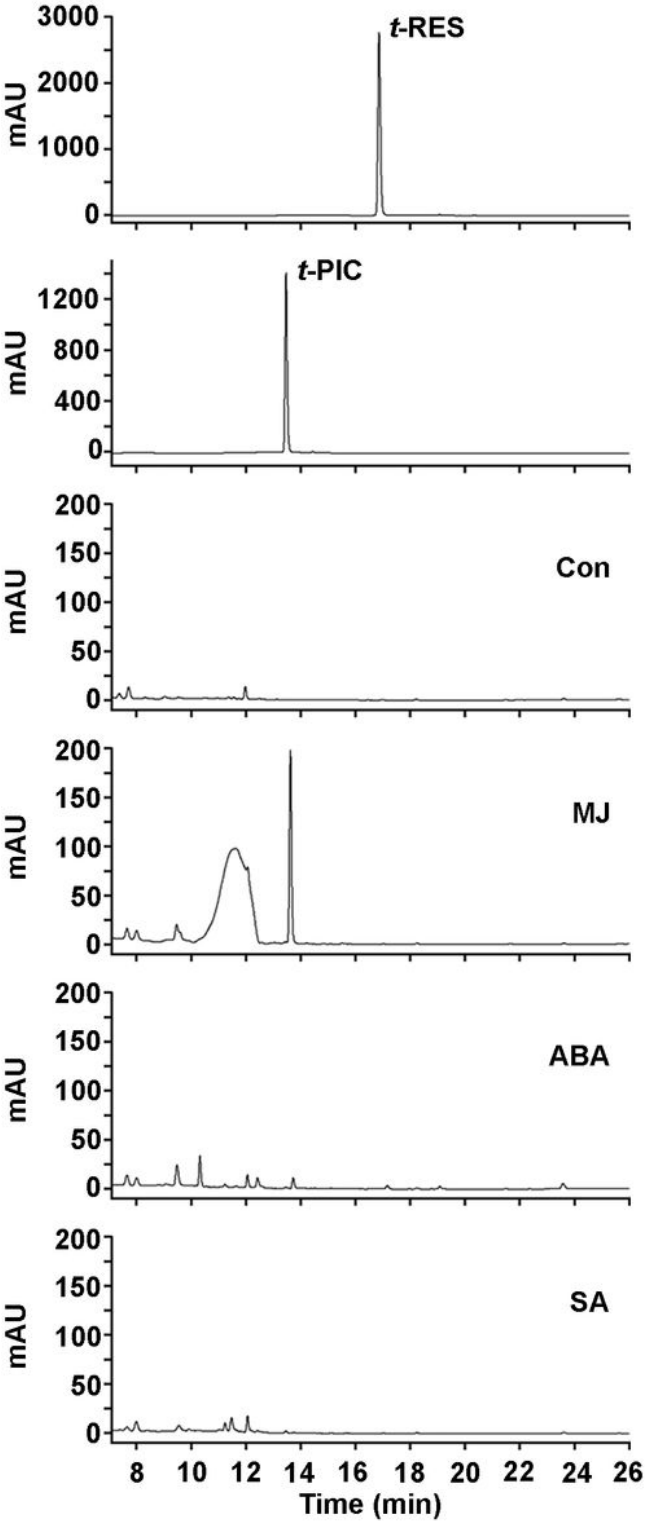

Figure 3

Effects of various elicitors on accumulation of stilbene compounds in grapevine cell cultures. The cells were pre-cultured for 7 days and harvested 2 days after elicitation with the indicated elicitors. Samples were extracted with methanol and subjected to HPLC analysis. HPLC analysis was performed using a C18 reverse-phase column at 300 nm. Chromatograms t-RES and t-PIC represent the authentic standards trans-resveratrol and t-piceid with retention times of 17.200 and 13.568 min, respectively. Con, untreated

control. Chromatogram MJ indicates the t-piceid production in the elicited cells 2 days after elicitation with 100 µM MJ.

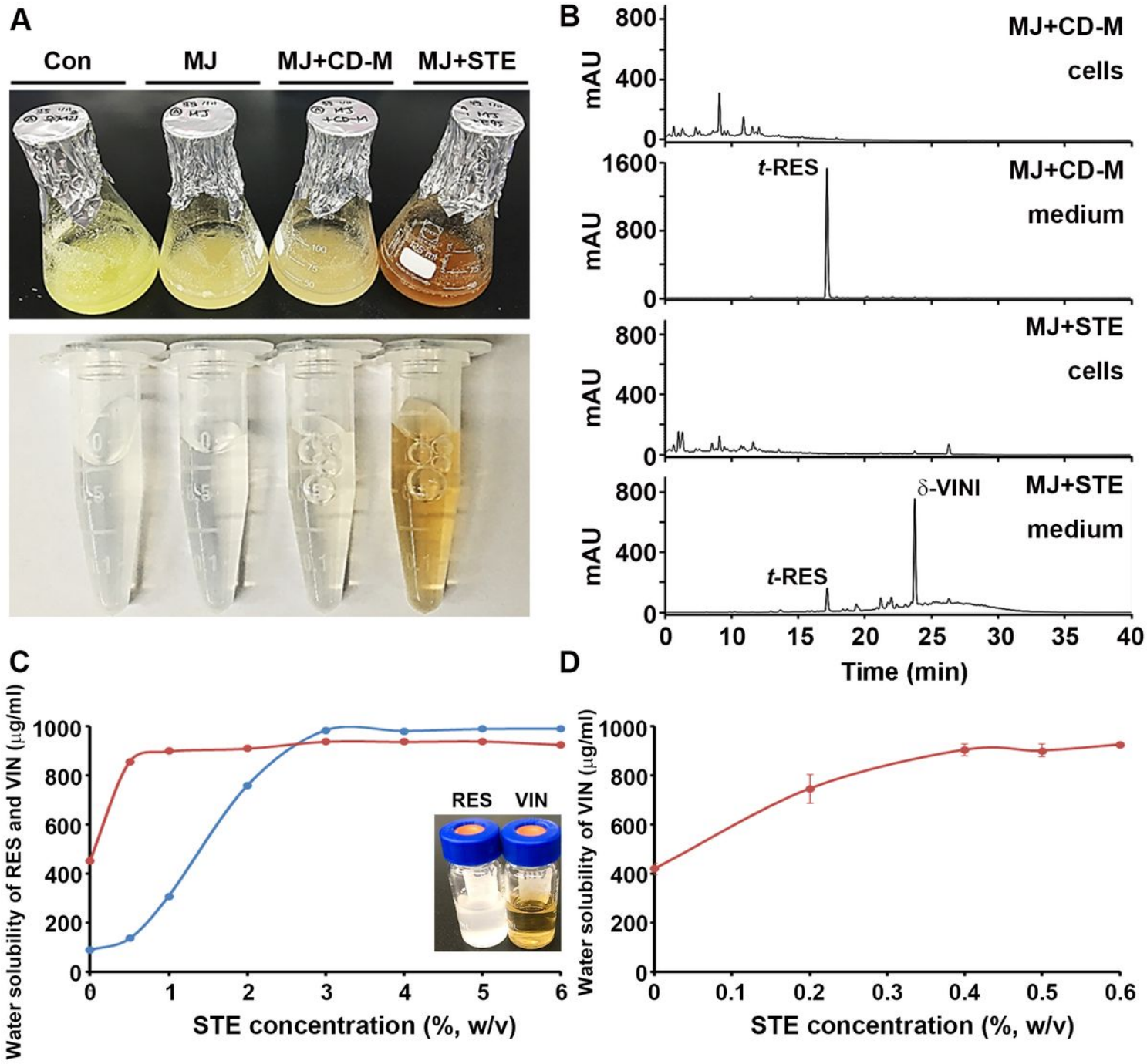

Figure 4

Extracellular production of trans-resveratrol and δ-viniferin in the grapevine cell culture medium. (A, B) Extracellular production of trans-resveratrol and δ-viniferin in grapevine cell culture medium after treatment with different solubilizers. Cells were pre-cultured for 7 days and harvested 5 days after co-application of 100 µM MJ and 50 mM CD-M or 50 mM STE as a solubilizer. (C, D) Water solubility enhancement of trans-resveratrol and δ-viniferin in STE solution. The solubility of trans-resveratrol (blue) and δ-viniferin (red) in aqueous solution with increasing STE concentrations (%, w/v) was determined.

(Insert) Images of trans-resveratrol (RES) and δ-viniferin (VIN) dispersion with 0.5% STE concentration (w/v). The culture medium of the elicited cells was extracted with ethyl acetate and analyzed by HPLC.

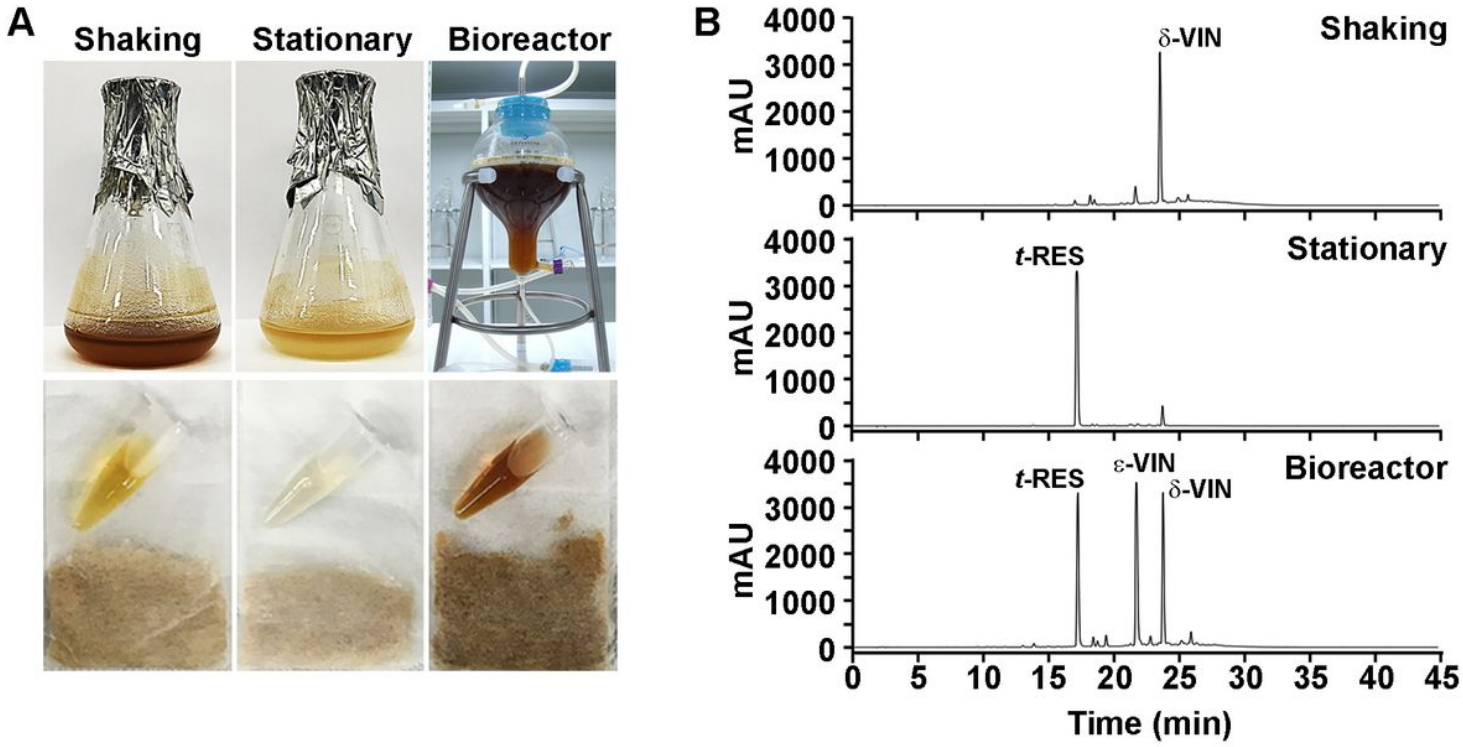

Figure 5

Conditional production of trans-resveratrol, δ-viniferin, and ε-viniferin in grapevine cell culture medium under different culture conditions. (A) Phenotypes of grapevine cell cultures 5 days after co-treatment with 100 μM MJ and 50 mM STE in flask (shaking or stationary) and bioreactor cultures. (B) HPLC chromatograms (300 nm) of ethyl acetate extract from the culture medium of the elicited cell cultures showing production of resveratrol and viniferin in flask and bioreactor cultures. For flask and bioreactor cultures of grapevine cells, cells were pre-cultured for 7 or 14 days in 500-mL Erlenmeyer flask and in a 3 L bioreactor, respectively, and harvested 5 days after co-treatment with 100 μM MJ and 50 mM STE.

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