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Squalestatin-induced production of taxol and baccatin in cell suspension culture of yew (*Taxus baccata* L.)

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Abstract: Various elicitors have already been reported to enhance the production of taxol and related taxanes. In this study, effects of a fungal metabolite, squalestatin, on production of taxol and baccatin III were studied. Expressions of 4 key involved genes, *ts*, *dbat*, *bapt*, and *dbtnbt*, in suspension cultures of *Taxus baccata* were also detected using qPCR. Results showed that application of squalestatin significantly increased taxol and baccatin III yields. Increased expressions of the genes were in accordance with measures of taxol and baccatin accumulations in cells and medium. Production of H_2O_2 has significant positive correlations with both gene expression and taxanes, indicating that the increase in H_2O_2 might be involved in the upregulation of the taxane production in yew under squalestatin treatment. Our results suggest that H_2O_2 is a key signaling component in the stimulation of taxane production in *T. baccata* cells induced by squalestatin.

Key words: Baccatin, cell suspension, H_2O_2, squalestatin, taxol

1. Introduction

Taxol (paclitaxel) is a potent antimitotic agent and an excellent active anticancer drug for the treatment of breast and ovarian cancers and AIDS-related Kaposi's sarcoma (Sgadari et al., 2000). Chemically, taxol is a diterpenoid alkaloid extracted from the bark of yew (*Taxus baccata* L.). The limited supply of this valuable drug from the bark of wild yews prompted intensive efforts to develop alternative ways of taxol production. Isolation from plantation-grown *Taxus* spp., biosynthesis in tissue culture systems, semisynthesis of taxol from the 10-deacetylbaccatin III readily available in the needles of yew, and total synthesis of taxol are some such notable efforts (Malik et al., 2011). Productions of paclitaxel and other related taxanes through large-scale cell cultures have been unstable and often of low yield. Different strategies, including application of precursors and elicitors, optimizing of cultural conditions, screening of high yielding cell lines, optimization of growth and production media, use of a 2-phase culture system, and immobilization, have been already tested to improve the yield of taxol in cultures of *Taxus* spp. (Malik et al., 2011).

Various defense responses such as signaling through elicitors, oxidative burst, peroxidation of lipids, and biosynthesis of phytoalexins or other secondary metabolites as defense compounds were reported by Mehdy (1994) as caused by application of fungal elicitors in cell cultures. An early event of plant response to different stress levels, which acts as a signal for the production of secondary metabolism, is the production of reactive oxygen species (ROS), also known as oxidative burst (Low and Merida, 1996).

Promotion of secondary metabolite production in other plant species through application of various fungal elicitors (including cell wall fragments, polysaccharides, oligosaccharides, and glycoproteins) has been reported (Holkova et al., 2010; Esmaeilzadeh Bahabadi et al., 2012; Prasad et al., 2013). Fungal elicitors were found to induce taxol production in cell suspension cultures of *T. chinensis* (Yu et al., 2002; Lan et al., 2003). Little is known about the use of fungal elicitors to enhance taxane production in *T. baccata* (Khosroushahi et al., 2006) or the expression profiles of the genes involved in the taxane biosynthetic pathways upon elicitation. It was claimed that activities of certain key enzymes in the biosynthesis pathway of taxanes may be induced through elicitation. It was shown that geranylgeranyl diphosphate synthase (GGPP synthase) and taxadiene synthase were induced

by methyl jasmonate (MeJ) treatment (Laskaris et al., 1999; Dong and Zhong, 2001). Furthermore, Onrubiab et al. (2010) showed that MeJ significantly increased the expression of *ts* and *bapt* genes, while vanadyl sulfate treatment just resulted in increased expression of the *bapt* gene.

This study aimed to evaluate the promotion of taxol and baccatin III production in cell suspension cultures of yew (*T. baccata*) by application of squalestatin as a fungal elicitor. To shed more light on the mechanisms involved in the biosynthetic pathway of taxanes in yew, H_2O_2 production and the expression of 4 key corresponding genes (*ts* at the beginning and *dbat*, *bapt*, and *dbtnbt* at the ending points of the pathway) under elicitation treatment was also assessed.

2. Materials and methods

2.1. Plant material and culture conditions

Young leaves were collected from adult *T. baccata* trees in Bula Forest (Chahar-dungeh, Mazandaran Province, Iran). Explants were surface-sterilized using 70% (v/v) ethanol for 30 s and sodium hypochlorite (10%) for 10 min, and were then rinsed 3 times with sterile water. Leaf explants were cut into segments of 0.5 cm in length; cultured on Gamborg's B5 medium supplemented with 2 mg/L naphthalene acetic acid, 0.2 mg/L 2,4-dichlorophenoxyacetic acid, 0.2 mg/L kinetin, 500 mg/L polyvinylpyrrolidone, 30 g/L sucrose, and 10 g/L agar; and kept for 5 months at 25 °C in darkness for callus induction.

Homogeneous calli were obtained from several subcultures in the same medium. Cell suspension cultures were initiated by suspending 1 g of fast-growing light-colored calli in 100 mL of medium in 250-mL Erlenmeyer flasks closed with aluminum foil and placed on a shaker at 110 rpm in the dark. Squalestatin (zaragozic acid) (Sigma-Aldrich, Inc.) was dissolved in 50 mM potassium phosphate (pH 7.5) and prepared as a concentrated stock solution. Two concentrations of squalestatin (0.1 and 1 μM) were added to the 21-day-old cell suspension cultures. Unelicited cell cultures were given similar volumes of potassium phosphate buffer and were used as the control.

2.2. Biomass accumulation and viability assay

The cells from a sampled shake flask were washed with a large amount of distilled water, filtrated under vacuum, and freeze-dried for 2 days. The dry cell weight was then determined. The viability of the cells during the experiment was determined by the trypan blue dye exclusion method. Briefly, 20-μL cell suspension samples were mixed with 20 μL of 0.1% trypan blue (prepared in PBS buffer) and incubated for 10 min. Cell viability was checked under light microscope.

2.3. H_2O_2 determination

The concentration of H_2O_2 was determined 1 day after elicitation with squalestatin, according to Alexieva et al. (2001). In 1.5 mL of trichloroacetic acid (0.1%), 50 mg of fresh cells was homogenized and centrifuged at 15,000 × *g* for 15 min. Next, 0.5 mL of the supernatant was added to 0.5 mL of 10 mM phosphate buffer (pH 7.0) and 1.0 mL of 1 M KI. After 1 h of reaction in darkness, the absorbance of the solution was measured at 390 nm, and the H_2O_2 concentration was calculated using a standard curve prepared with known concentrations of H_2O_2.

2.4. RNA extraction and real-time RT-polymerase chain reaction

Total RNA was extracted using the QIAzol Lysis Reagent (QIAGEN). Briefly, 100 mg of cells was homogenized in liquid nitrogen and dissolved in 1 mL of reagent buffer. After the addition of 0.2 mL of chloroform and centrifugation at 12,000 × *g* for 15 min at 4 °C for phase separation, the aqueous phase containing RNA was obtained; an equal volume of isopropanol was added and the mixture was centrifuged at 12,000 × *g* for 10 min at 4 °C. Supernatant was then obtained, and the resulting RNA pellet was washed twice with 1 mL of 75% ethanol and then centrifuged at 7500 × *g* for 5 min at 4 °C. The obtained dry RNA pellet was dissolved in diethylpyrocarbonate-treated water. Quantiscript Reverse Transcriptase (QIAGEN) was used for synthesis of cDNA with oligo-dT and random primers according to the manufacturer's protocol.

RT-PCR assays were performed using the Rotor-Gene Q apparatus (QIAGEN). Real-time RT-PCRs of 18S rRNA (housekeeping gene) and *dbat*, *ts*, *bapt*, and *dbtnbt* cDNAs were performed using specific primers (Table) and SYBR Premix Ex Taq (TaKaRa). The thermocycler program for all reactions included an initial denaturation step at 95 °C for 3 min, followed by 40 amplification cycles consisting of denaturation at 94 °C for 30 s, annealing at 60 °C for 10 s, and extension at 72 °C for 25 s. Real-time PCR experiments were carried out at least 4 times, with the threshold cycle (Ct) determined in triplicate. Differences in Ct value between an unknown sample and the calibrator are expressed as fold-changes relative to the calibrator sample. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta Ct}$ method.

2.5. Taxol and baccatin III measurement

Taxanes were extracted separately from freeze-dried cells and the culture medium as described previously (Wang et al., 2001). Briefly, lyophilized cells were ultrasonicated with 5 mL of methanol for 40 min at room temperature. Methanol extracts were evaporated to dryness, redissolved in 1 mL of methanol, and filtered through a 0.22-μm filter for subsequent analysis. Samples of cell-free medium were extracted with methylene chloride (1:1, v/v). The organic phase was removed from the aqueous phase and

Table. Sequences of RT-PCR primers.

Designation	Primer sequence	Product size (bp)
18S rRNA	F: 5'-CCGCGGTAATTCCAGCTCCAA-3'	113
	R: 5'-GAGGGCCAGTGCACACCGAGTA-3'	
ts	F: 5'-TTCGCACGCACGGATACG-3'	97
	R: 5'-TTCACCACGCTTCTCAATTCG-3'	
dbat	F: 5'-GGGAGGGTGCTCTGTTTGTG-3'	230
	R: 5'-GCCACGATGACCTCTGGAAC-3'	
bapt	F: 5'-TAAGCACTCTACAACAACAATGG-3'	111
	R: 5'-GCATGAACATTAGTATCTTGATTCC-3'	
dbtnbt	F: 5'-GTTGAGCTGGAAGTGTGGCA-3'	124
	R: 5'-GCCACGATGACCTCTGGAAC-3'	

concentrated in a rotary. The residue was dissolved in 1 mL of methanol and filtered before being subjected to high-performance liquid chromatography (HPLC) analysis. Quantifications of taxanes were performed using an HPLC system (Agilent 1100) based on the methods of Wang et al. (1999). Twenty milliliters of each sample was injected into a reverse-phase column (Zorbax Eclipse XDB-C18, 150 × 4.6 mm). The mobile phase consisted of isocratic (at constant concentration) acetonitrile and water (35:65, v/v), and the flow rate was 1 mL/min. Apparatus wavelength was adjusted to 228 nm for both baccatin III and taxol. Identification of unknown peaks was based on retention time and UV absorption spectral comparison with known standards of taxol (Sigma) and baccatin (Sigma). The area under the peaks is proportional to the amounts of taxanes.

2.6. Statistical analysis

Experiments were replicated 3 times, and statistical significance was measured using the 1-way analysis of variance test. Least significant different mean comparison was used to test significant differences between treatments at the 5% level.

3. Results

3.1. Effect of squalestatin on cell growth index and production of H_2O_2, taxol, and baccatin III

Cell viability remained high (≥82%) in both treatments. Application of 0.1 or 1 μM squalestatin had no significant effect on cell growth index (data not shown). As shown in Figure 1, both concentrations of squalestatin (0.1 and 1 μM) stimulated H_2O_2 production in *T. baccata* cell suspension culture by 1.5 times over the control 1 day after elicitation. Total taxane production in samples elicited by squalestatin is presented in Figure 2. The 2 concentrations of squalestatin (0.1 and 1 μM) increased
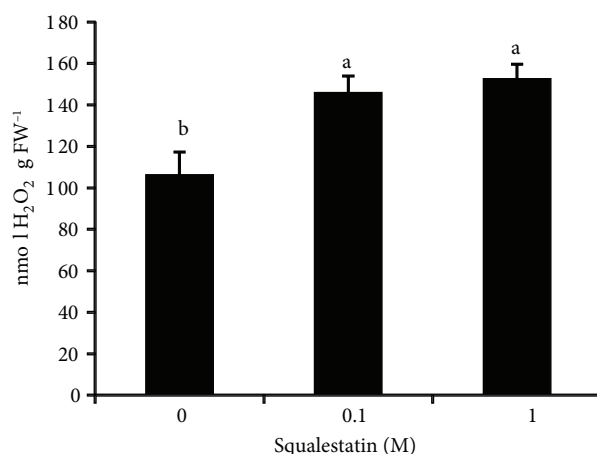

Figure 1. The effects of squalestatin on H_2O_2 production in cell suspension cultures. Vertical bars indicate SE of the mean for n = 3.

baccatin III contents by 1.99 and 1.64 times compared to the control, respectively. Similarly, a significant increase in taxol contents was observed under squalestatin treatment at both concentrations (0.1 and 1 μM) compared to the control (2.47 and 1.87 mg/L, respectively). Average contents of baccatin in cells treated with squalestatin are shown in Figure 3A; this content was highest (1.33 mg/L) in the 0.1 μM squalestatin treatment. Since the total baccatin produced in this treatment was high, intracellular baccatin is expected to be high for this treatment.

Results showed that there were significant differences in terms of extracellular baccatin accumulation between the 2 concentrations of squalestatin (Figure 3B). The highest amount of extracellular baccatin was observed with the 1 μM squalestatin treatment (1.6 mg/L),
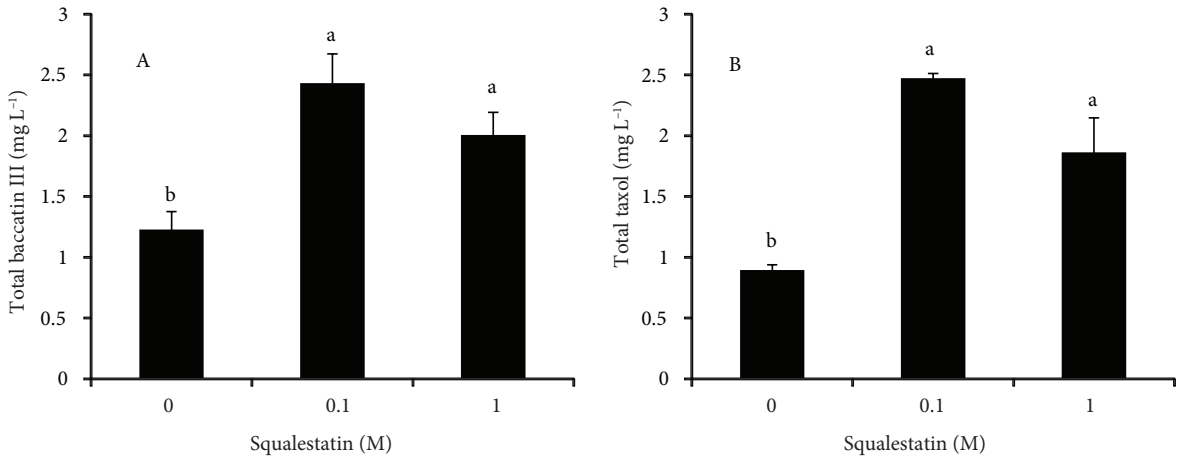
Figure 2. The effects of squalestatin on total baccatin III (A) and total taxol (B) production in cell suspension cultures. Vertical bars indicate SE of the mean for n = 3.
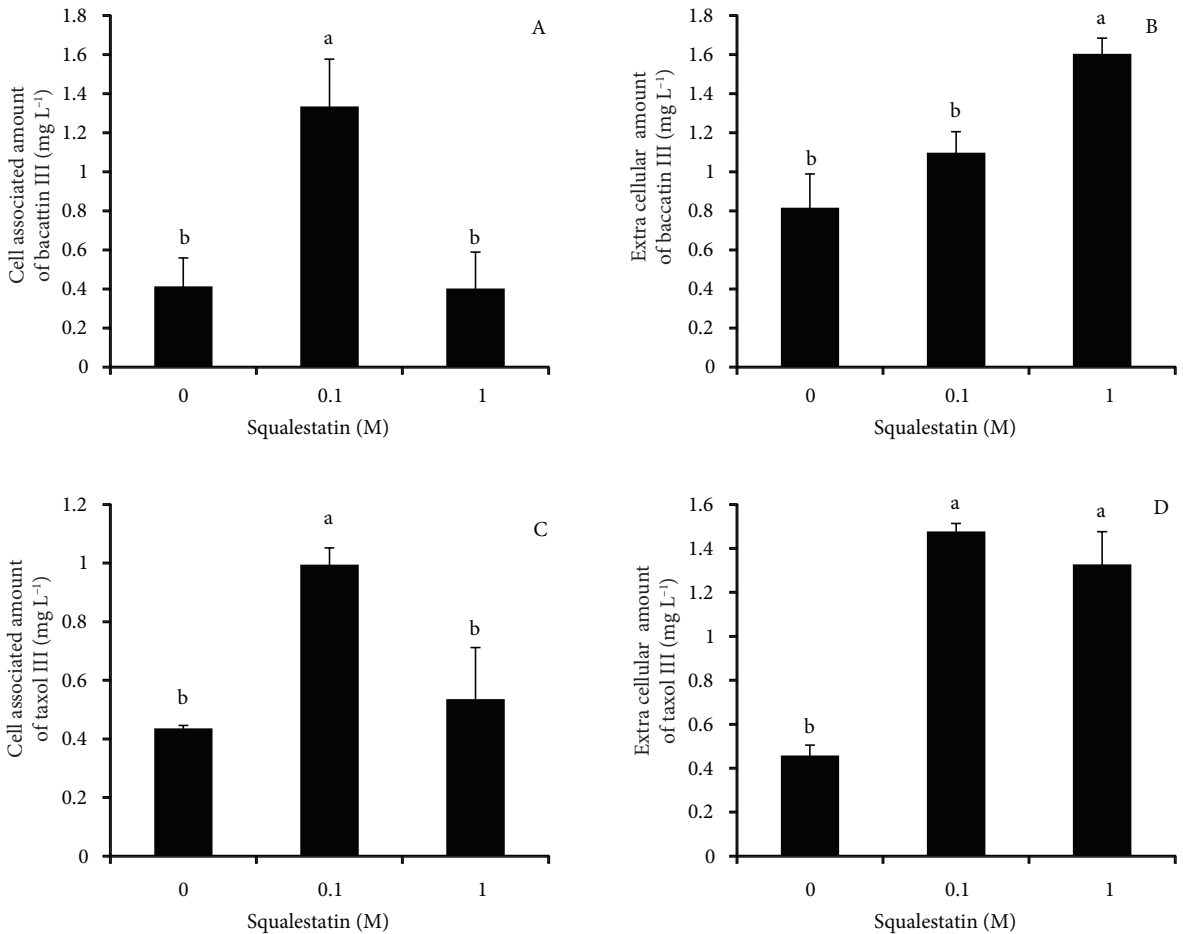

Figure 3. Effect of squalestatin on cell-associated baccatin III (A), extracellular baccatin III (B), cell-associated taxol (C), and extracellular taxol (D) in *T. baccata* suspension cultures. Vertical bars indicate SE of the mean for n = 3.

corresponding to 79% of total baccatin production in this treatment. It is concluded that 1 μM (1.6 mg/L) squalestatin was more effective in removing baccatin from the cells. Cell-associated taxol was increased significantly in cell suspension culture upon treatment by 0.1 μM squalestatin (Figure 3C), but no significant changes in cell-associated taxol were observed upon treatment with 1 μM squalestatin compared to the control. Maximum extracellular taxol (1.32 mg/L) was measured in 1 μM squalestatin (1.6 mg/L), corresponding to 71.26% of total taxol in this treatment.

3.2. Effect of squalestatin on expression of key genes in taxanes' metabolic pathways

Changes in transcription levels of *ts*, *dbat*, *bapt*, and *dbtnbt* genes were evaluated in this study using the qPCR technique to determine the mechanism of squalestatin action on taxane biosynthesis. Taxadiene synthase (EC 4.2.3.17, alternative name: taxa-4(5),11(12)-diene synthase), which catalyzes the cyclization of the ubiquitous isoprenoid intermediate GGPP to taxa-4,11-diene, the

parent olefin with a taxane skeleton, is considered as the first step in the taxol biosynthesis pathway. Production of 10-deacetylbaccatin III is catalyzed via taxane 2α-O-benzoyltransferase, which is then converted to baccatin III by 10-deacetylbaccatin III-10-O-acetyltransferase (DBAT, EC 2.3.1.167) (Walker et al., 2000). *bapt* is another key gene encoding baccatin III, 13-O-3-amino-3-phenylpropanoyl transferase; it is responsible for the cleavage of the lateral chain to baccatin III in the final biosynthetic steps, leading to production of taxol. 3'-N-Debenzoyl-2-deoxytaxol-N-benzoyltransferase (DBTNBT) ligates a benzoyl CoA group to 3'-N-debenzoyl-2-deoxytaxol, leading to production of 2'-deoxytaxol.

Expressions of these 4 key corresponding genes in the taxanes' biosynthetic pathway were measured for 2 concentrations of squalestatin (Figure 4). The results showed that expression of the *ts* gene was increased by 4 times in cells elicited with squalestatin at 0.1 or 1 μM. Expression of *dbat* was also significantly increased by 8 and 2 times compared to the control 1 day after elicitation
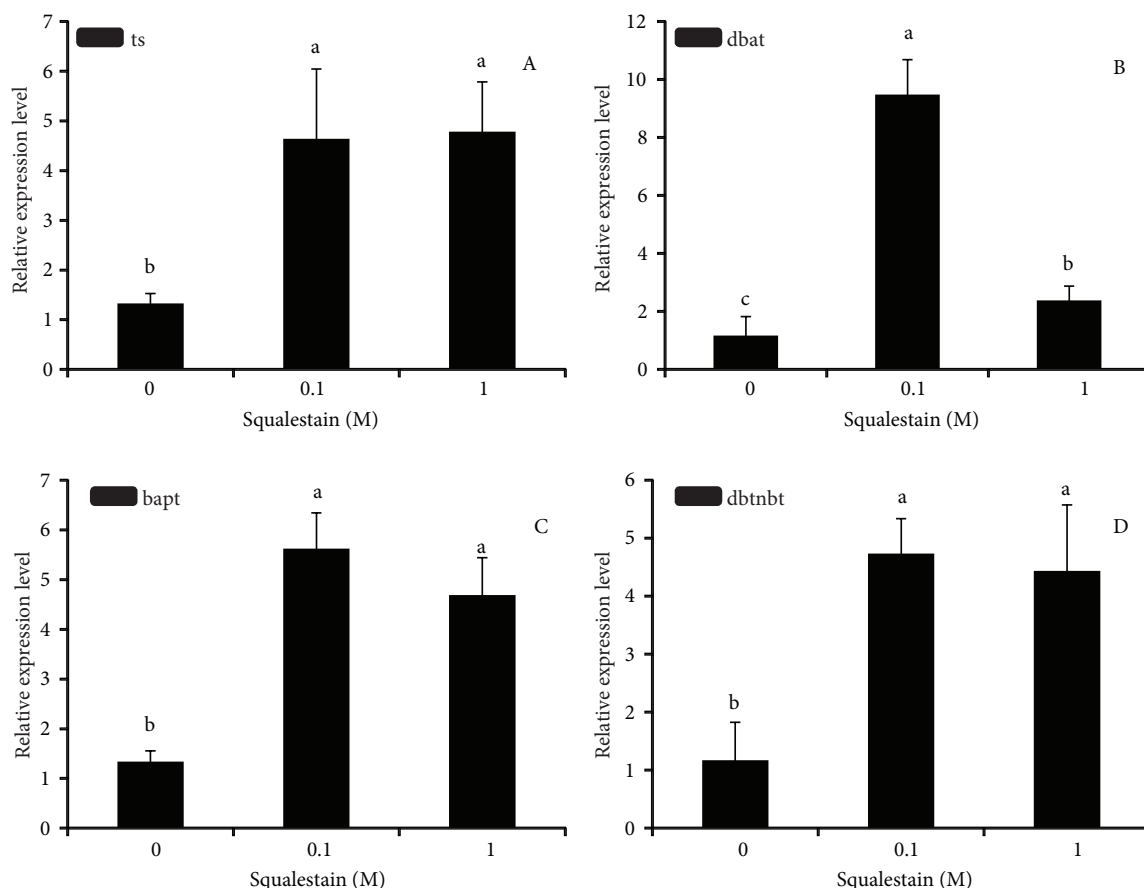

Figure 4. Quantitative analysis of expressions of key genes in the biosynthesis pathway of taxol. Relative gene expression is shown for *ts* (A), *dbat* (B), *bapt* (C), and *dbtnbt* (D). The data are expressed as relative gene expression to 18S rRNA and normalized to a control of untreated cells (0). Vertical bars indicate SE of the mean for n = 3.

by squalestatin at 0.1 and 1 µM, respectively. Expressions of both *bapt* and *dbtnbt* significantly increased (~4 times) in elicited cells compared to the control for both concentrations of squalestatin.

The H_2O_2 values obtained were linearly correlated to gene expression and to total baccatin and total taxol (R^2 = 0.99, *ts*; R^2 = 0.88, *bapt*; R^2 = 0.95, *dbtnbt*; $P \leq 0.05$; and R^2 = 0.78, baccatin; R^2 = 0.75, taxol; $P \leq 0.05$, respectively).

4. Discussion

The necessity for more detailed understanding of taxol metabolism and molecular mechanisms of enzymatic controls in reactions leading to taxol biosynthesis has already been highlighted (Vongpaseuth and Robert, 2007). Transcription of the genes corresponding to the enzymes located in terminal steps of the metabolic pathway was reported to be less than that of the genes in the beginning of pathway upon MeJ elicitation (Nims et al., 2006). Nims et al. (2006) suggested that these terminal steps of the pathway were the potential rate-influencing steps in the paclitaxel biosynthetic pathway. Most recent studies on plant cell cultures have focused on the control of secondary metabolite production via identification of key steps capable of restriction of the velocities in biosynthetic pathways. Conversion of geranylgeranyl pyrophosphate to the universal precursor of diterpenoids, which is catalyzed by taxadiene synthase enzyme, is reported as a slow step compared to other steps in the biosynthetic pathway (Roberts and Shuler, 1997). Our results showed that the expression of corresponding gene *ts* was increased in the beginning steps of the taxol biosynthetic pathway via squalestatin treatment.

Our results obtained for elicited cells of yew were similar to those obtained by Esmaeilzadeh Bahabadi et al. (2012) and Satheesan et al. (2012), where application of fungal elicitors enhanced gene expression of secondary metabolites' biosynthetic pathways. Few reports are available, to date, on the effects of squalestatin on gene expressions in plant biosynthetic pathways. Wentzinger et al. (2002), for example, evaluated the effect of squalestatin on the expression of the *HMGR* gene in *Nicotiana tabacum* cell suspension culture and showed that its expression was most increased after 24 h of treatment. Regarding the production of taxanes in *Taxus* spp. cell suspension cultures, which is often enhanced by MeJ via activation of the genes encoding the relevant biosynthetic enzymes (Nims et al., 2006; Onrubiab al., 2010), the question that arises that how squalestatin enhanced the expression of genes involved in taxanes' biosynthesis pathway. It is known that ROS may induce expression of many defense and secondary metabolite biosynthetic genes, such as sesquiterpene cyclases and PAL (Mehdy, 1994). Allen and Tresiki (2000) found that H_2O_2 might serve as an

intermediate signal regulating synthesis of secondary metabolites in plant–fungal interactions. In this study, treatment of *T. baccata* cell cultures with squalestatin increased the production of H_2O_2.

H_2O_2-mediated lipid peroxidation could initiate the octadecanoid pathway, leading to biosynthesis of jasmonate (JA), which has been reported to function in the induction of plant secondary metabolites (Thoma et al., 2003). It is known from many previous studies that endogenous JA accumulated after fungal elicitor treatment of cell cultures (Gundlach et al., 1992; Blechert et al., 1995). Endogenous jasmonic acid was reported by Blechert et al. (1995) to be temporarily increased in *Agrostis tenuis* suspension cultures after fungal elicitation. This was regarded as a specific effect, since JA did not resynthesize under other stresses like light and heavy metals and cold or heat shock stresses.

In this study, treatment of yew cells with squalestatin initially resulted in the induction of H_2O_2 production, and occurrence of oxidative stress in elicited cells of yew was confirmed by the significant positive correlations between H_2O_2 and both gene expression and total baccatin and total taxol. Moreover, our previous work (Jalalpour et al., 2014) demonstrated that squalestatin-induced secondary metabolite production was mediated through a JA-dependent signaling pathway in yew cells by the increasing of lipoxygenase activity during the 48 h after elicitation. The present work, however, showed that H_2O_2 was induced by squalestatin 24 h after elicitation, and so elicitor-induced JA was directly mediated by H_2O_2 and acted as a downstream signal molecule in the stimulation of taxane production in *T. baccata* cells.

Biosynthesis of taxol and squalene-derived products shared the same isoprenoid biosynthetic pathway upon the precursor farnesyl diphosphate (FPP) (Huang et al., 2007). Decreasing the carbon flux through competitive pathways is shown to be one of the useful approaches to improvement of the desired secondary metabolism (Oksman-Caldentey and Inze, 2004). Regulation of squalene synthase, an important branch-point enzyme in isoprenoids' biosynthetic pathway, may result in more production of secondary metabolites in yew. Squalene synthase is a potential regulatory factor capable of controlling carbon flux into the biosynthesis pathway of taxol and squalene-derived products. Squalestatin specifically inhibits squalene synthase, which may lead to the direction of FPP away from sterol biosynthesis and towards the synthesis of other desired isoprenoids (Figure 5). Song (2003) showed that disruption of sterol biosynthesis by squalene synthase resulted in a remarkable accumulation of FPP in an erg9 mutant strain of *Saccharomyces cerevisiae*. Besides FPP, the increase of isopentenyl diphosphate and GGPP was also observed when rat liver cells were treated with
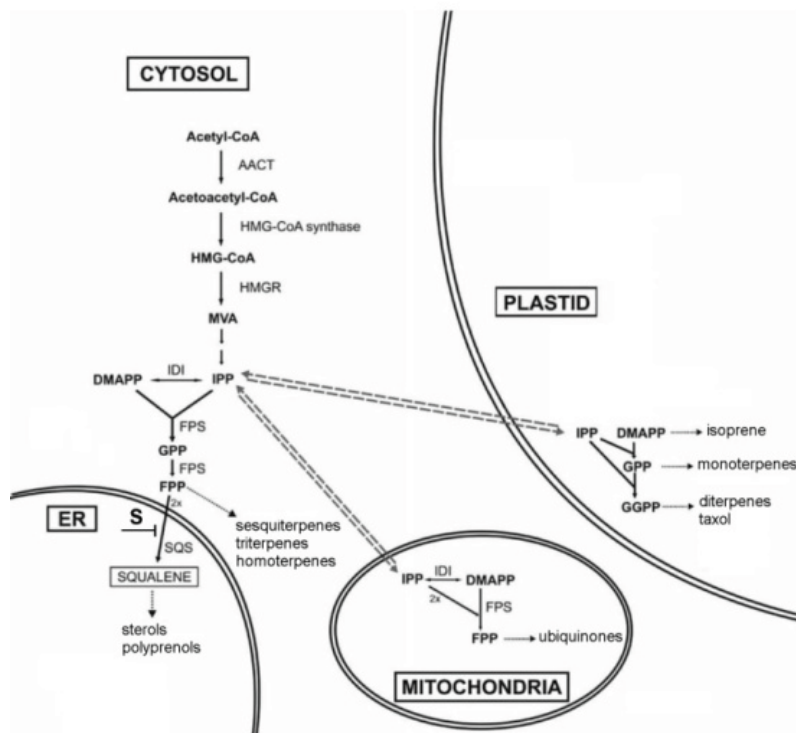
Figure 5. Isoprenoid biosynthetic pathway. Inhibition of squalene synthase by squalestatin (S). FPP, farnesyl diphosphate; FPS, FPP synthase; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; AACT, acetoacetyl CoA thiolase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, HMG-CoA reductase; IDI, isopentenyldiphosphate isomerase; DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl diphosphate; MVA, mevalonate; SQS, squalene synthase; ER, endoplasmic reticulum (Spanova and Daum, 2011).

zaragozic acid, a potent inhibitor of squalene synthase (Keller, 1996). Similar effects were also observed in plants (Fulton et al., 1995). Squalestatin may therefore effectively inhibit squalene synthase in the competitive pathways of isoprenoids and sterols, leading to utilization of substrates for the accumulation of taxanes.

The elicitor of interest might retain maximum production along with high excretion of products to the medium. Some reports claim that ≥90% taxol was excreted to the medium (Hirasuna et al., 1996; Srinivasan et al., 1996), while there are, on the other hand, reports that regard taxol as an intracellular substance (Wickremesinhe and Arteea, 1993). It should be noted that taxol excretion to the medium is dependent on the type and conditions of the medium, and on plant genotype. This study showed that the concentration of the elicitor had prominent effects of baccatin and taxol excretion to the medium.

Results of this study showed similar changes (4-fold) in transcription levels of *ts*, *bapt*, and *dbtnbt* genes upon treatment with both the squalestatin concentrations. Although baccatin (the product of the corresponding

enzyme to the *dbat* gene) was similarly increased 2-fold with both treatments, the transcription level of the gene was significantly different at 0.1 (8 times) and 1 mM (2 times) concentrations of squalestatin. Increase in production of baccatin III might be due to the effect of squalestatin on the gene expression at the beginning steps of the metabolic pathway, and the inhibitory effect of squalestatin on squalene synthase enzyme may direct the precursors towards production of nonsterol compounds in cell suspension cultures of this plant.

It is concluded that application of squalestatin in yew cell culture induced a cascade of events including accumulation of H_2O_2 and endogenous MeJ and suppressed squalene synthase activity, which was correlated with the overexpression of key genes, resulting in remarkable increases in taxane production.

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