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ORIGINAL ARTICLE

Effect of coronatine on synthesis of cephalotaxine in suspension cells of *Cephalotaxus mannii* and its transcriptome analysis

Liu-Yan Wang[1] · Qiao Zhang[1,2] · Zi-Qi Wang[1] · Yong-Cheng Li[1]

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Abstract

To improve the production of cephalotaxine and explore its biosynthesis pathway in the suspension cells of *Cephalotaxus mannii*, the cell suspension cultures were separately treated with elicitors, including coronatine (0.5, 1.0, 2.0 μmol/L) and methyl jasmonate (100 μmol/L), and then analyzed their transcriptome differences. The results showed that coronatine could improve the content of cephalotaxine in suspension cells of *C. mannii*. The highest product (6.75 mg/L) appeared in the coronatine treatment of 1.0 μmol/L, which was higher than that of methyl jasmonate treatment (4.29 mg/L) and control (3.14 mg/L). The key enzyme of the shikimic acid pathway, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DS) activity increased after 24 h of adding elicitors above. Transcriptome analysis indicated that the phenylpropane pathway, shikimic acid pathway, glucose metabolism process, phenylalanine, and tyrosine synthesis pathway were involved in the synthesis of cephalotaxine. In addition, glucose-6-phosphate dehydrogenase (G6PDH), hexokinase, phenylalanine ammonialyase (PAL), and Cytochrome P450, etc. were found out to be closely related to the cephalotaxine accumulation. It could be concluded that coronatine promoted the synthesis of cephalotaxine by affecting the expression of some enzymes, especially DS in the synthesis pathway of cephalotaxine.

Key message

Coronatine (1.0 μmol/L) led a yield of 6.75 mg/L cephalotaxine, which was 2.15 times of the control. The transcriptome analysis indicated DS played an important role in cephalotaxine biosynthesis.

Keywords *Cephalotaxus mannii* · Cephalotaxine · Coronatine · Methyl jasmonate · Transcriptome

Abbreviations

CDS	Coding sequence
COR	Coronatine
CT	Cycle threshold
DEGs	Differential expression genes
DS	3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase
E4P	Erythrite 4-phosphate
FW	Fresh weight
G6P-2Na	D-Glucose-6-Phosphate 2Na
G6PDH	Glucose-6-phosphate dehydrogenase
KT	Kinetin
MJ	Methyl jasmonate
NAA	1-Naphthylacetic acid
NADP	Nicotinamide adenine dinucleotide phosphate
PEP	Phosphoenolpyruvate
PVP	Polyvinyl pyrrolidone
TCA	Trichloroacetic acid
TTC	Triphenyltetrazolium chloride
VB1	Vitamin B1

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✉ Yong-Cheng Li
lyc2360@sina.com

[1] College of Food Science and Engineering, Hainan University, Haikou 570228, People's Republic of China

[2] College of Food and Biological Engineering, Hezhou University, Hezhou 542899, People's Republic of China

Introduction

Cephalotaxine is a benzylisoquinoline alkaloid isolated from *Cephalotaxus* plants. In China, cephalotaxine is mostly extracted from *Cephalotaxus mannii*, and has been a hot of research in the field of anticancer drugs (Meng et al. 2017). Due to the meager yield of cephalotaxine extracted from

plants and the limited natural resources, it is not enough to support the clinical application. In order to improve the production of cephalotaxine, the predecessors have done a lot of work. The main methods, semi-synthetic and biological synthesis was applied to produce cephalotaxine. Compared with the semi-synthetic method, bio-extraction of cephalotaxine from the plant suspension cells cultures is more environmentally friendly. The secondary metabolites in plant cell cultures can be promoted by many adopted strategies, such as elicitation, metabolic regulation, optimizing the culture conditions, adding possible precursors, etc. (Li and Jiang 2019; Verpoorte et al. 2002). Among these methods, the addition of methyl jasmonate, salicylic acid and some biological products of fungi and other elicitors is the most common and effective (Cusido et al. 2014). Coronatine is composed of one molecule containing an alpha-amino acid and another molecule of coronamic acid containing a polyketone structure (Ichihara et al. 1977). As an effective elicitor, coronatine was utilized to increase the secondary metabolites production in many plant cells, such as caffeic acid, isoferulic acid, ρ-coumaric acid, sinapic acid, and phytosterols in *Lemna paucicostata* (Kim et al. 2017).

The synthesis of cephalotaxine was involved in the glycolysis pathway, pentose phosphate pathway, shikimic acid pathway and the subsequent reactions (Fig. 1). In these metabolic pathways, erythrose-4-phosphate, 3-deoxy-D-arabino-heptulose-7-phosphate, phenylalanine, tyrosine and 1-phenethyltetrahydroisoquinoline are the precursors for cephalotaxine production (Gitterman et al. 1980; Parry et al. 1980). Although the synthesis pathway of cephalotaxine has been roughly explored, the key enzymes in its synthesis pathway still have not been confirmed. The development of omics technology has provided a very convenient and effective method for determining the key enzymes in various biosynthetic pathways (Zhao et al. 2018). A large amount of mRNA data in a certain biological stage can be obtained from transcriptome analysis through a small number of samples. For non-model plants, reassembly techniques for RNA sequencing can determine unsequenced RNA (Kakumanu et al. 2012; Yue et al. 2018). These technologies above provide the possibility for exploring the biosynthetic pathways of plant secondary metabolites.

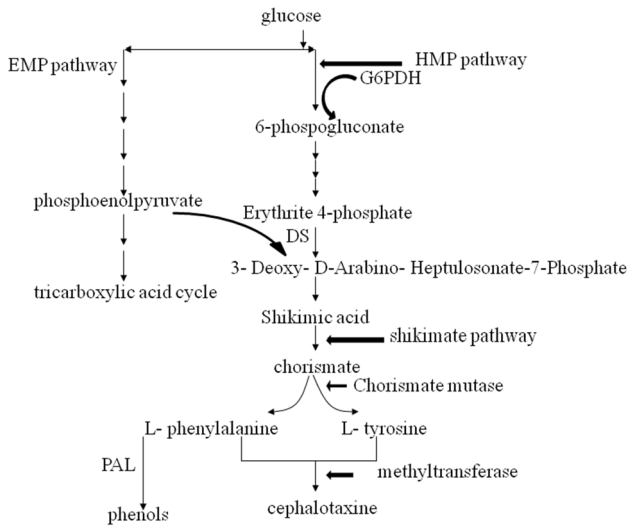

Fig. 1 The schematic for the biosynthesis pathway of cephalotaxine and related enzymes in *C.mannii*

In this study, suspension cells of *C. mannii* were treated with coronatine to promote the synthesis of cephalotaxine. Besides, as an efficient elicitor, coronatine can disturb the normal metabolism of plant cells, and thereout generate large differentially expressed genes (DEGs) for transcriptome analysis. By analyzing transcriptome data, the metabolic pathways of cephalotaxine in *C. mannii* suspension cells and its key enzymes were explored.

Materials and methods

Suspension culture of *C. mannii* cells

Cephalotaxus mannii callus induced from its leaves were cultured on MS medium and subcultured once a month in darkness under 26 °C. To obtain suspension cultures, 20 g of 30-day callus was transferred to 100 mL MS liquid medium in a 500 mL flask and cultured on a shaker at 100 rpm and 26 °C. Subsequently, plant suspension cell cultures were subcultured by replacing 60 mL used medium with equal volume of fresh medium every 2 weeks. Cell suspension cultures were subcultured for five generations before the test (Li and Jiang 2019).

Elicitor treatment

Dissolving coronatine (Sigma) with deionized water, sterile coronatine solution was added to the suspension cultures on day 15 of culture, the final concentration of coronatine in the cell suspension solution was 0.5, 1.0, and 2.0 μmol/L, respectively. Simultaneously, the sterilized methyl jasmonate (MJ, Tokyo Chemical Industry) solution (dissolved with ethanol) was also supplemented to the cell suspension cultures and the final concentration was of 100 μmol/L.

Measurement of cell viability

Triphenyltetrazolium chloride (TTC) solution (6 g/L) was used in the determination of cell viability (Trevors et al. 1983). In addition, cells dyed with 0.4% (w/v) trypan blue were observed under the microscope. The dark blue cells mean low active cells or dead cells, and slight-colored cells mean high activity cells.

Assay of sugar consumption

The total sugar in the cultures was determined by phenol–sulfuric acid method. Briefly, 1 mL of sample was mixed with 1 mL of 5% (w/v) phenol, followed by slowly adding 5 mL of sulfuric acid into the mixture. After keeping at room temperature for 30 min, the absorbance was calculated at 480 nm.

Assay of glucose-6-phosphate dehydrogenase (G6PDH) activity

The G6PDH activity was determined based on the study of Yu et al. (2004). One unit (U) is defined as the amount of enzyme required for the change in absorbance of 0.01 at 340 nm every 5 min before and after the reaction. Cell enzyme activity is expressed by enzyme units per gram of wet cells (U/g).

Assay of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DS) activity

The DS activity determined according to the study of Mccandliss et al. (1978). Changes in absorbance of 0.1 at 549 nm in a minute were recorded to calculate the enzyme activity unit (U).

Determination of cephalotaxine

Extracellular and intracellular product of the culture were extracted according to Li et al. (2014). ZORBAX Elipse Plus C18 column (4.6 × 250 mm, 5 µm, Agilent) on the Agilent 1260 system was chosen to determine the content of cephalotaxine. The mobile phase was composed of methanol and ammonium acetate with a ratio of 45:55 (v/v), and the flow rate was set at 1.0 mL/min. After injecting 10 µL of the sample, the elution was monitored in an ultraviolet detector at the absorbance of 280 nm.

Transcriptome analysis of *C. mannii* suspension cells

After adding elicitors for 48 h, 0.1 g fresh cells were obtained. Gene Denovo Biotechnology Co., Ltd. (Guangzhou, China) performed the transcriptome analysis. High-quality reads were selected through fragment filtering, then compared with rRNA and reference genes. These screened reads used for bioinformatics analysis, mainly including quantification of gene abundance, differential expression genes (DEGs), sample relationship, GO enrichment, and pathway enrichment.

RT-qPCR analysis

Total RNA was extracted from 0.1 g fresh suspension cells by a RNA extraction kit (Tiangen). RNA quality was demonstrated by gel electrophoresis analysis. Using the same amount of RNA as the template, cDNA was synthesized according to the instructions of the cDNA first strand synthesis kit (Tiangen).

The gene expression of G6PDH, DS and Hexokinase of suspension cells treated with elicitors was confirmed by real-time qPCR. The 18s rRNA gene of *C.mannii* was used as the reference gene (Sun et al. 2019). According to the coding sequence (CDS) of those genes above, gene-specific primers were designed using Primer Primer 5 (Table 1).

PCR was performed by BIO-RAD CFX Connect Real-Time System. Briefly, 0.4 µL of cDNA template was added to 2× SuperReal PreMix Plus (Tiangen) of 10 µL, 10 µM of each primer and ddH_2O to a final volume of 20 µL. The reactions were carried out at 95 °C for 15 min, followed by 40 cycles at 95 °C for 10 s and then 60 °C for 20 s and extended at 72 °C for 20 s. The cycle threshold (CT) values were set automatically by the system, and the changes of each gene were calculated using the equation $2^{-\Delta\Delta CT}$, where ΔΔCT = (CT target gene − CT reference) Sample X − (CT target gene − CT reference) control.

Statistical analysis

Each group in the experiment had three independent replicates, and the results were tested by the Tukey test and expressed by the average ± standard deviation. The statistical analysis was finished by using SPSS Statistic 23.0, $P < 0.05$ was considered as a significant difference.

Table 1 Primers used in real-time RT-PCR

Target	Primer	Sequence
18srRNA	Forward	GCTGAAATGAGCACGAGGTCC
	Reverse	CACGAGATAAAGTTTGCCCACA
G6PDH	Forward	GCTCATTCAAAATCATCTCCT
	Reverse	GCTTCATTATGTATTCGCAAA
DS	Forward	GCAGTGCGTCGTGCTGGGCTTA
	Reverse	CTGGGTGGCTCCCTTCTTG
Hexokinase	Forward	CGCCAGGTTGTCTGTGTGT
	Reverse	CGTGATGGTGATTGGGTCC

Results

The effect of coronatine on the growth of *C. mannii* suspension cell and its product

The different concentrations of 0.5, 1.0, and 2.0 µmol/L coronatine were added into *C.mannii* suspension cultures on day 15. Changes in biomass, cell viability, and sugar consumption of cultures were shown in Fig. 2. On the 30th day, the biomass of cells treated with 1.0 µmol/L coronatine declined by 2.3% while control didn't change significantly, and it was declined by 6.4% when 2.0 µmol/L coronatine was added (Fig. 2a). The result confirmed that high concentration coronatine would depress cell growth.

The growth of suspension cells was closely related to cell viability. It could be observed that different degrees of cell viability decline were appeared after adding 0.5, 1.0, and 2.0 µmol/L coronatine compared with the control (Fig. 2b). On the 20th day, the viability of cells treated with 0.5, 1.0, and 2.0 µmol/L coronatine was decreased to 4.41, 6.72 and 2.77 OD_{492}/g, respectively, while that of the control was 14.08 OD_{492}/g. The results demonstrated that coronatine would exhibit high cytotoxicity when its addition dose was over 2.0 µmol/L. Meanwhile, the residual sugar content in suspension liquid was tested and presented in Fig. 2c. It was obvious that coronatine could reduce glucose consumption of cultures, which was corresponding to the results of low cell viability caused by coronatine. On the 25th day, the residual sugar concentration in the cultures added with 0.5, 1.0, and 2.0 µmol/L coronatine was 5.39, 7.47, 8.53 g/L, while the control was 0.74 g/L. It reflected that coronatine had an inhibitory effect on the growth and the viability of suspension cells.

In order to further investigate the effect of inducers on cell viability, the microscopic images of suspension cells stained with trypan were recorded (Fig. 3). Some suspension cells were found dead after adding coronatine for 1 day (the 16th day after inoculation). On the 20th day, there were hardly survival cells in 2.0 µmol/L coronatine treated cultures, but viable cells were still observed in 1.0 µmol/L coronatine treatment. So, cells treated with 1.0 µmol/L coronatine maintained higher viability and kept more integral morphology than other treatments.

In regard to the effects of coronatine on the cephalotaxine production, the highest production of cephalotaxine (6.75 mg/L) was achieved in 1.0 µmol/L coronatine treatment (Table 2), which was more than twice of the control (3.14 mg/L). Additionally, the results showed that 2.0 µmol/L coronatine treatment cells had the lowest yield of cephalotaxine (0.47 mg/L). Obviously, it showed that the high concentration of elicitor added did not mean the more yield of cephalotaxine. The optimum concentration of coronatine for cephalotaxine synthesis in *C.mannii* suspension cells was 1.0 µmol/L in this study.

The comparative study of coronatine and methyl jasmonate (MJ)

MJ was an efficient elicitor for secondary metabolite accumulation in plant tissue and cell culture. In our previous work, 100 µmol/L MJ was an optimal dose for cephalotaxine production in *C. mannii* suspension cultures (data not reported). To confirmed the coronatine mechanism on cephalotaxine production in *C. mannii* suspension cultures, the comparative study of coronatine of 1.0 µmol/L and 100 µmol/L MJ was completed in the following work.

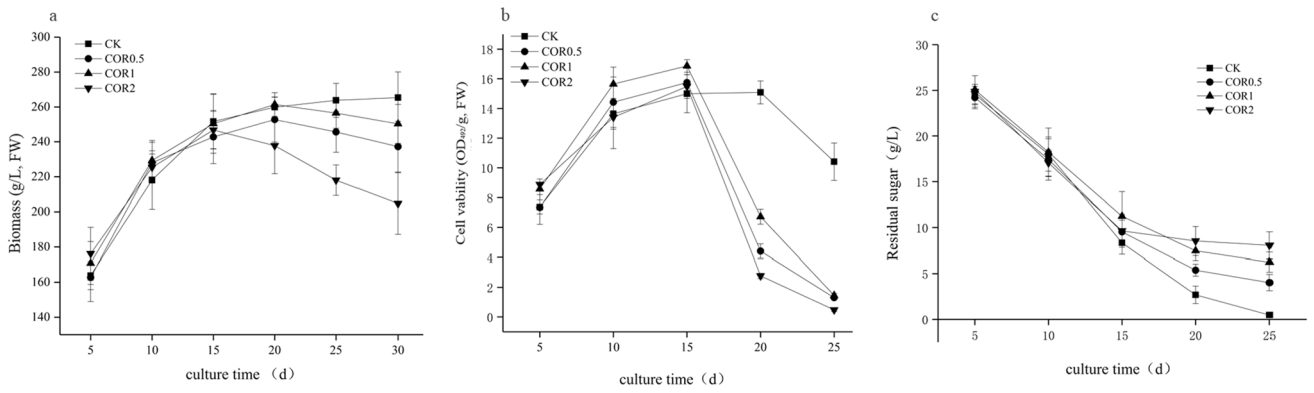

Fig. 2 Effects of coronatine on the biomass (**a**), cell viability (**b**) and residual sugar (**c**) of *C. mannii* suspension culture. Coronatine was added on the 15th day. CK represented the control, COR0.5, COR1, COR2 represented the treatment with 0.5, 1.0, and 2.0 µmol/L coronatine, respectively. All cell weight means fresh weight (FW)

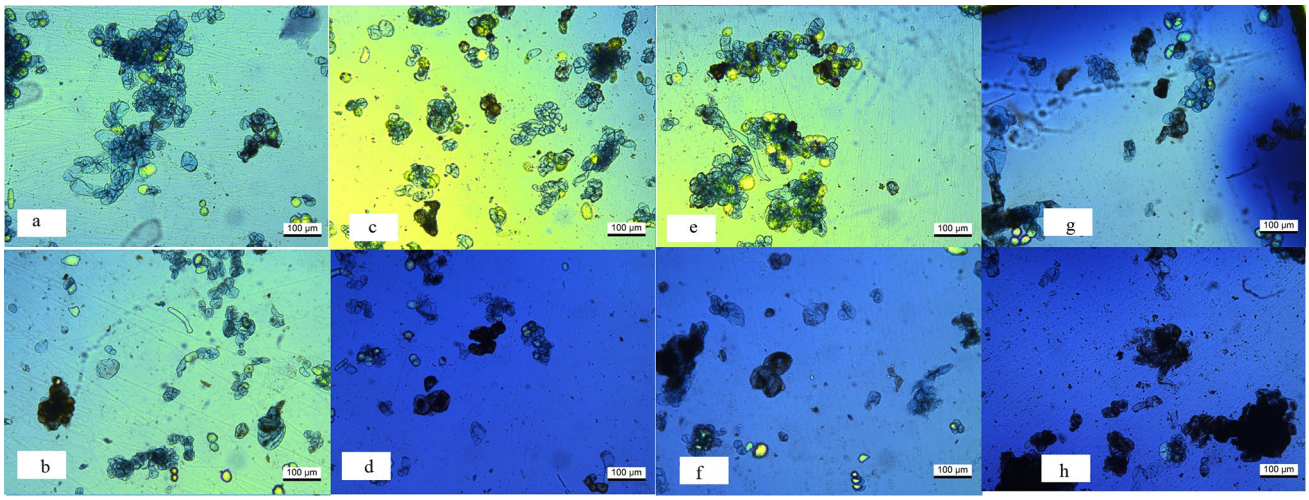

Fig. 3 Microscopic images of suspension cells during culture. The unstained cells meant active, while cells dyed blue were dead or low activity. **a**, **b** The morphology of control cells on the 16th and 20th day after inoculation. **c** (16 days) and **d** (20 days) were of cells treated with 0.5 µmol/L coronatine. **e** (16 days) and **f** (20 days) were of cells treated with 1.0 µmol/L coronatine. **g**, **h** were of cells treated with 2.0 µmol/L coronatine on the 16th day and 20th after inoculation

Table 2 Effect of coronatine on the production of cephalotaxine in *C. mannii* suspension cultures

Treatment	Cephalotaxine (mg/L)			Extracellular (mg/L)	Intracellular (mg/L)	Release rate (%)
	Harringtonine	Homoharringtonine	Total*			
CK	1.26 ± 0.06^{a}	1.88 ± 0.15^{b}	3.14 ± 0.48^{b}	$1.77 \pm 0.31^{a,b}$	$1.37 \pm 0.12^{a,b}$	56.4
0.5 µmol/L Cor	2.89 ± 0.03^{b}	2.63 ± 0.44^{b}	5.52 ± 1.18^{c}	2.52 ± 1.47^{b}	2.99 ± 1.51^{b}	45.7
1 µmol/L Cor	3.01 ± 0.34^{b}	3.74 ± 0.54^{c}	6.75 ± 0.73^{d}	4.09 ± 1.10^{c}	1.84 ± 0.96^{c}	60.6
2 µmol/L Cor	0.31 ± 0.03^{a}	0.16 ± 0.02^{a}	0.47 ± 0.06^{a}	0.39 ± 0.05^{a}	0.08 ± 0.03^{a}	83.0

Data were means ± SD (n = 3)

*Total cephalotaxine is the sum of harringtonine and homoharringtonine

Different lowercase letters in the same column indicated a significant difference at the level of $P < 0.05$, Tukey test was used to test the statistical significance

Effect of coronatine and MJ on *C. mannii* suspension cell growth

When 1.0 µmol/L coronatine and 100 µmol/L MJ was added into *C. mannii* suspension cultures on day 15, the changing trend in biomass treated with coronatine was not significantly different from that of control. But treatment with MJ was the opposite. The biomass of cells treated with MJ was decreased by 8.8% while control increased on day 20 (Fig. 4a). It is obvious that coronatine had no apparent inhibitory effect on *C. mannii* cells growth compared with MJ.

The cell viability with culture days was shown in Fig. 4b. Compared with the control, the cell viability all declined after adding coronatine or MJ. On the 20th day, the viability of cells treated with 1.0 µmol/L coronatine was decreased to 6.72 OD_{492}/g and 1.47 OD_{492}/g by MJ treatment, while that of the control was 14.08 OD_{492}/g. MJ had more inhibitions on cell viability than coronatine.

Meanwhile, the residual sugar content in suspension liquid was tested and presented in Fig. 4c. It was evident that coronatine or MJ all reduced glucose consumption, which corresponded to the results of cell viability. On the 25th day, the residual sugar concentration in the cultures added with MJ was 8.26 g/L, and that by added with 1.0 µmol/L coronatine was 7.47 g/L. But the control was 0.74 g/L, which reflected that coronatine and MJ all had a great inhibitory effect on glucose consumption.

Microscopic images of suspension cells stained with trypan were recorded (Fig. 5). On the 20th day, there were hardly survival cells in MJ treated cultures, but some viable cells were still observed in coronatine treated cultures. So, cells treated with 1.0 µmol/L coronatine maintained higher viability and kept more integral morphology than MJ treatment.

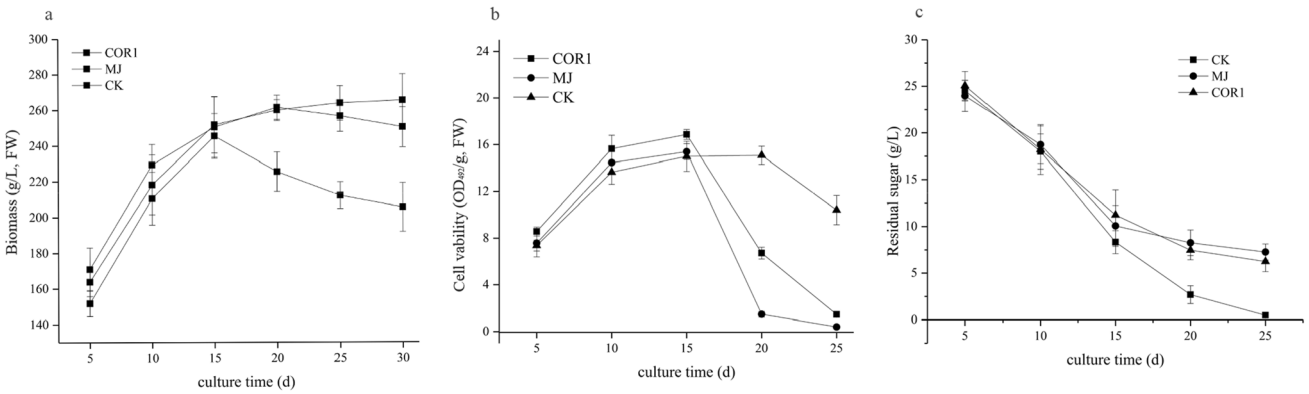

Fig. 4 Effects of elicitor on the biomass (**a**), cell viability (**b**) and residual sugar (**c**) of *C. mannii* suspension cultures. COR and MJ were added on the 15th day. CK represented the control. MJ and COR1 represented the cells treated with MJ and 1.0 μmol/L COR, respectively

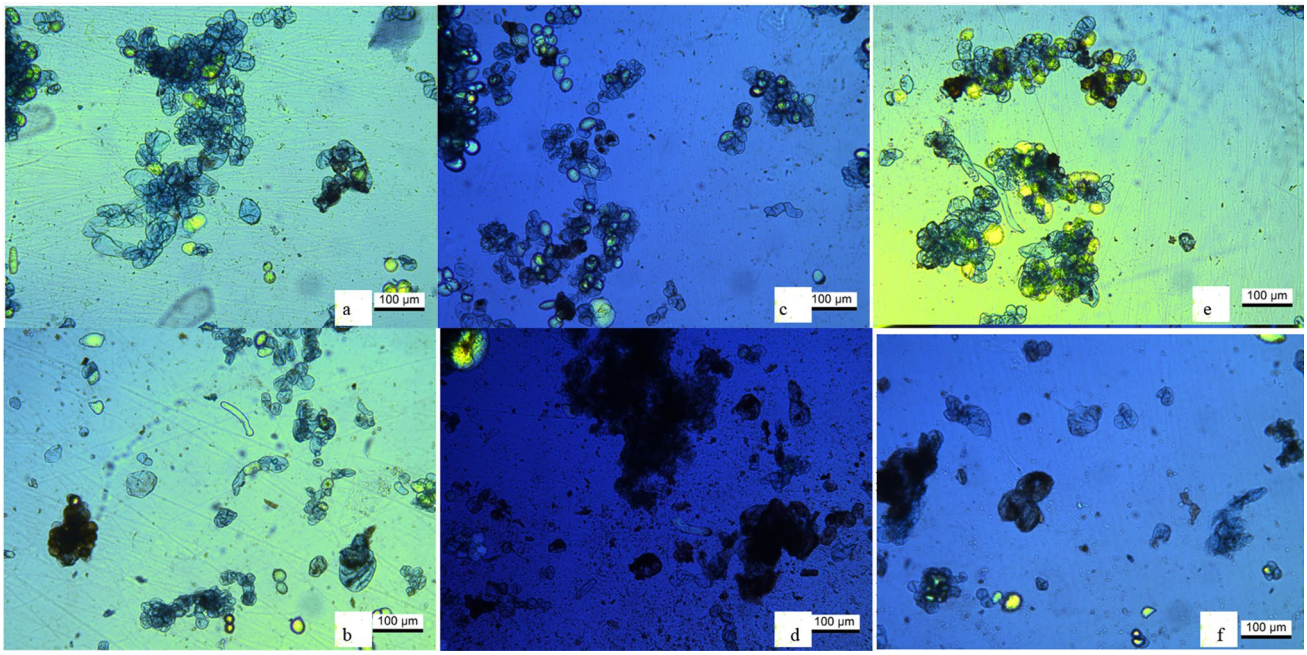

Fig. 5 Microscopic images of suspension cells during culture. The unstained cells meant active, while cells dyed blue were dead or low activity. **a**, **b** The morphology of cells of the control on the 16th and 20th day after inoculation. **c** (16 days) and **d** (20 days) were of cells treated with 100 μmol/L MJ. **e** (16 days) and **f** (20 days) were of cells treated with 1.0 μmol/L coronatine

Table 3 Effect of coronatine and MJ on the production of cephalotaxine in *C. mannii* suspension cultures

Treatment	Cephalotaxine (mg/L)			Extracellular (mg/L)	Intracellular (mg/L)	Release rate (%)
	Harringtonine	Homoharringtonine	Total			
Cor 1	3.01 ± 0.34	3.74 ± 0.54	6.75 ± 0.73	4.09 ± 1.10	1.84 ± 0.96	60.6
MJ	2.25 ± 0.61	2.04 ± 0.61	4.29 ± 0.63	2.68 ± 1.21	1.61 ± 1.45	62.5

Data were means ± SD (n = 3). Cor 1 means 1 μmol/L coronatine treatment, and MJ means treatment with 100 μmol/L MJ

Cephalotaxine production in *C. mannii* suspension cultures treated with coronatine and MJ

When 1.0 µmol/L coronatine was added, 6.75 mg/L cephalotaxine was detected in suspension cultures, and there were also 4.29 mg/L cephalotaxine produced by cells treated with 100 µmol/L MJ (Table 3). Thus, coronatine was a better elicitor for cephalotaxine formation in suspension cells of *C. mannii* than MJ.

Effects of coronatine and MJ on key enzymes of cephalotaxine synthesis pathway

In the synthesis of cephalotaxine, the precursor, erythrose-4 phosphate, was produced through the pentose phosphate pathway, which was involved in a key enzyme G6PDH (Stincone et al. 2015). After adding coronatine and MJ for 48 h, all the G6PDH activity was increased (Fig. 6a), but the enzyme activity increased by coronatine (70.8 U/g) was higher than that by MJ (58.4 U/g), which means coronatine can induce more production of erythrose-4 phosphate and was in favor of subsequent cephalotaxine synthesis. DS was the key enzyme in the synthesis of shikimic acid, its activity of cells treated with elicitors was improved compared with the control. Cells treated with coronatine had higher DS activity. After adding coronatine for 48 h, the activity upgraded from 7.2 to 21 U/g. Under the same condition, DS activity was increased from 8.5 to 15.6 U/g by MJ (Fig. 6b).

Differentially expressed genes and metabolism pathways analysis

Differentially expressed genes (DEGs) of cells treated with different elicitors shown in Table 4. A total of 11,682 DEGs was screened from CK-vs-COR1, and there were 16,125 DEGs in CK-vs-MJ; 11,454 DEGs were found in MJ-vs-COR1. The pathway enrichment analysis of DEGs indicated that the synthesis of cephalotaxine was mainly involved with glycolysis pathway, pentose pathway, phenylpropane pathway, tyrosine and phenylalanine metabolism (Fig. 7). Among these pathways, the pentose pathway could produce erythrose-4-phosphate, and tyrosine and phenylalanine were the precursors for the synthesis of cephalotaxine (Ni et al. 2016).

Related gene expression for cephalotaxine synthesis

The expression level of genes in the KEGG pathway analysis indicated by fragments per kilobase of exon model per

Table 4 Statistical analysis of expression difference

Difference analysis	Up-regulated expressed genes	Down-regulated expressed genes	Total number of DEGs
CK-vs-COR1	3849	7833	11,682
CK-vs-MJ	3827	12,298	16,125
MJ-vs-COR1	7905	3549	11,454

CK means the control, MJ means the treatment of adding 100 µmol/L MJ, COR1 means the treatment of 1.0 µmol/L coronatine

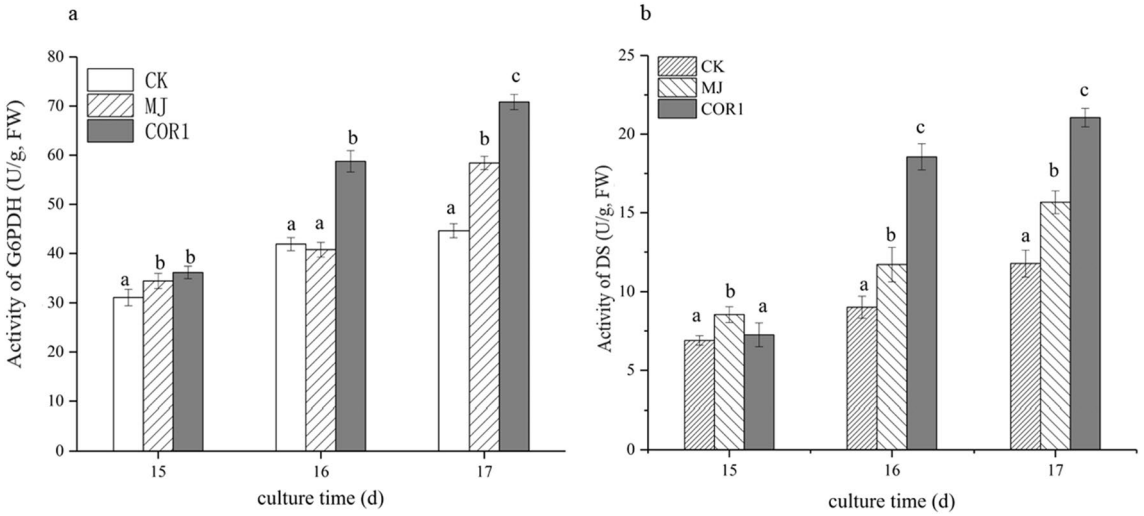

Fig. 6 The effects of elicitors on G6PDH (**a**) and DS (**b**) activity of *C. mannii* suspension cells. CK represented the control. MJ and COR1 represented the cells treated with 100 µmol/L MJ and 1.0 µmol/L coronatine, respectively. Data were mean ± SD (n = 3). Different lowercase letters on the same day indicated a significant difference at the level of $P < 0.05$

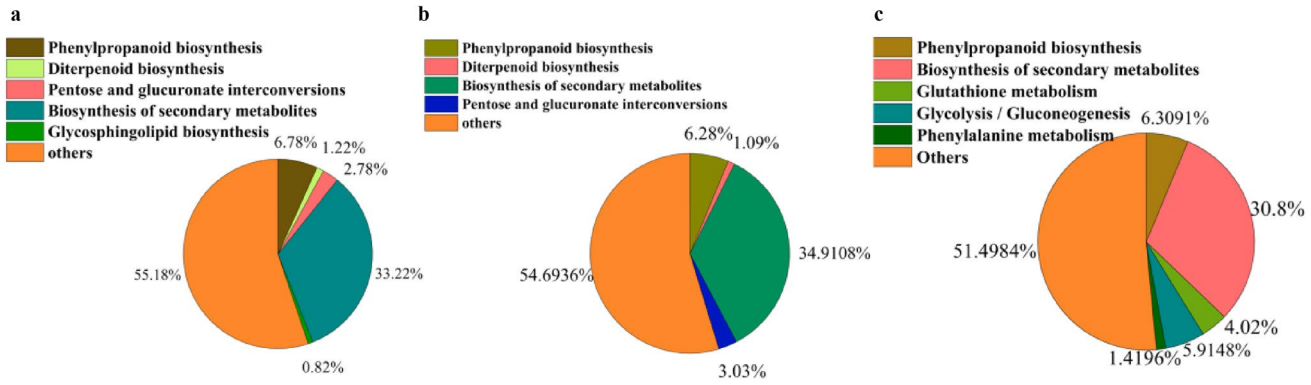

Fig. 7 The enrichment pathways analysis of differentially expressed genes. In the pie chart, **a**, **b**, and **c** represented DEGs enrichment pathway analysis of CK-vs-COR1, CK-vs-MJ, and MJ-vs-COR1, respectively. CK represented the control. MJ, COR1 represented the cells treated with 100 µmol/L MJ and 1.0 µmol/L coronatine, respectively

Table 5 Gene expression differences of cephalotaxine-related enzymes

	MJ-vs-CK	COR1-vs-CK	COR1-vs-MJ
DS	2.9↑	4.5↑	–
PAL	7.4↑	31.1↑	8.3↑
G6PDH	4.02↓	–	4.11↑
Hexokinase	2.78↑	5.15↑	2.01↑
Cytochrome P450	6.78↑	8.36↑	–
Sorbitol dehydrogenase	3.35↑	–	–
Isocitrate dehydrogenase	12.62↑	–	–
Phenylpropionyl-CoA ligase	1.65↑	–	–
Chorismate mutase	2.14↑	2.5↑	–
Chalcone synthase	–	6.43↑	–
Pyruvate dehydrogenase	–	11.17↑	–
Alcohol dehydrogenase	–	13.38↑	–
Phosphoenolpyruvate kinase	–	3.28↑	–
Cinnamoyl-CoA reductase	–	–	5.75↑
Glutamate decarboxylase	–	–	8.15↑
Sesquiterpene synthase	–	–	7.27↑
Diterpene synthase	–	–	2.36↑
Anthocyanin reductase	–	–	10.37↑
Pyruvate decarboxylase	–	–	4.59↑
Caffeoyl-CoA *O*-methyltransferase	–	–	2.27↑
S-norcoclaurine synthase 1	–	2.7↑	–
Laurate oxidase	3.33↓	6.97↓	–
Aspartate aminotransferase	1.05↓	2.24↓	–

↑ means up-regulation, ↓ means down-regulation, and numbers indicated up- or down-regulation multiples of the gene expression level (FPKM). COR1 means the treatment of 1.0 µmol/L coronatine

million mapped fragments (FPKM) in this report. Compared with the control, the expression level of the phenylalanine ammonialyase (PAL) gene in the MJ-treated cells up-regulated by 7.4 times, and that of the DS gene and isocitrate dehydrogenase gene separately up-regulated by 2.9 and 12.6 times (Table 5). In cells treated with coronatine, phenylalanine ammonialyase, pyruvate dehydrogenase, hexokinase, DS, phosphoenolpyruvate kinase, chalcone synthase, alcohol dehydrogenase, and polyphenol oxidase genes transcription levels were all increased by 2–31 times compared with control. In COR-vs-MJ, Cinnamoyl-CoA reductase, and pyruvate decarboxylase were found to up-regulated in the COR-treated cells. Besides, gene transcription levels of other enzymes, including glutamate decarboxylase, diterpene

synthase, anthocyanin reductase, also up-regulated in coronatine treatment compared with MJ treatment. Interestingly, among some critical enzymes involved in cephalotaxine synthesis, e.g., PAL, DS and G6PDH, the gene level of PAL and DS all increased in MJ and COR treatment. But, there was no obvious change about G6PDH gene expression, even exhibited down expression in MJ treatment.

RT-qPCR analysis

Three enzymes closely related to cephalotaxine synthesis, the expression level of DS, G6PDH, and hexokinase gene were verified by RT-qPCR after adding elicitors for 48 h. Figure 8 indicated that the expression level of DS and hexokinase enhanced greatly in all treatment especially in coronatine treatment. But there were not obvious changes about G6PDH in MJ treatment and only a small increase in coronatine treatment, which proved again that coronatine was better for cephalotaxine synthesis than MJ.

Discussion

Different concentrations of coronatine resulted in different quantities of products. The key to determining its effect was the concentration of coronatine added in the culture system. Uppalapati previously found that coronatine affected various hormone pathways of tomato, and produced cytotoxic effects on cells (Uppalapati et al. 2005). These results were similar to the researches that a high concentration of coronatine had a negative effect on cell viability and product formation (Fig. 2 and Table 2). Sánchez-Pujante revealed that the induction of antibacterial substances and defense-related proteins induced by coronatine could increase the production of metabolites in broccoli cells, and stated the elicitation effect of lower concentration coronatine (0.5 µmol/L) was better than the higher concentration (Sánchez-Pujante et al. 2020). Our microscopic examination results confirmed that high concentration coronatine caused cell death and even the autolysis of cells (Fig. 3). In this study, 1.0 µmol/L coronatine was an optimal concentration for cephalotaxine production in *C. mannii* suspension cells.

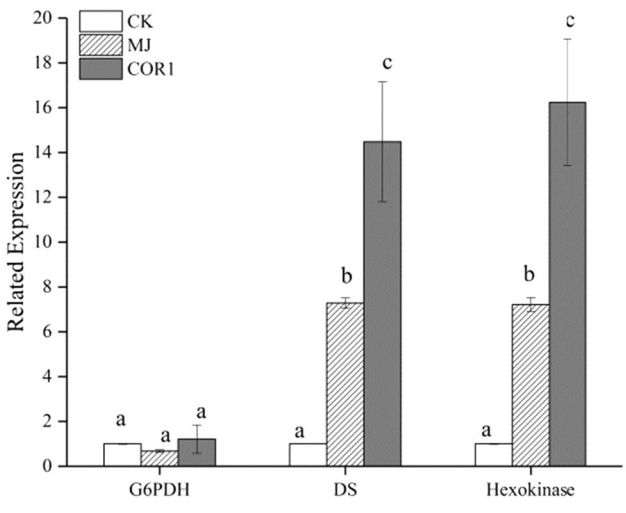

Fig. 8 The expression of different genes measured by RT-qPCR. Different lowercase letters in the same column indicated a significant difference at the level of $P < 0.05$

The cell growth curves of control (Fig. 4) showed that the logarithmic period of *C. mannii* cell growth was between day 10 to day 20, and stationary phase at day 20 to day 25, which indicated day 15 is in mid-log-phase. Only when sufficient primary metabolites accumulated in plant cells, it can get a high yield of the secondary metabolites (Li et al. 2020). The production of secondary metabolite happened at the end of the term linear stage or stable period. Therefore, the addition time of elicitor in this study was set on 15th day after inoculation, which meets with the many previous reports, such as in verbascoside production by *Buddleja cordata* Kunth cell, the optimum time of elicitor addition was at the middle to the late term of the logarithmic period (Arano-Varela et al. 2020). Although using elicitor was effective means for improving secondary metabolite production in the plant cell, it can induce cell death or decrease cell activity. According to present results in Figs. 4 and 5, MJ and coronatine all decreased the cell activity, but coronatine treatment maintained a better cell activity compared with MJ treatment, which means coronatine was more beneficial to the accumulation of products compared with MJ and that meets the results of cephalotaxine yields in Table 3. Thus it could be concluded that coronatine was a more suitable elicitor than MJ for cephalotaxine synthesis. In Onrubia's study, *Taxus media* cells treated with coronatine produced more paclitaxel and related taxanes, and the yield of paclitaxel in the coronatine treatment was 3.26 times that of MJ (Onrubia et al. 2013). But in our study, the product of coronatine treatment was only 1.6 times that of MJ (Table 3).

The production of secondary metabolites usually accompanied by the response of plant cells to external stress (Thakur et al. 2018). These responses and secondary metabolites accumulation are often involved in some common enzymes. Previous studies had shown that G6PDH was involved in the production of secondary metabolites in plant cells. Elicitors could increase the production of metabolites by promoting the activity of G6PDH (Randhir et al. 2004). Because when the cells were damaged caused by elicitors, G6PDH could improve the antioxidant capacity of cells and increased cell activity due to more NADPH produced by G6PDH (Yu et al. 2005). However, in coronatine treatment, the results of G6PDH activity (Fig. 6a) did not well match that of its gene expression (Table 5 and Fig. 8), which may be infer that the regulation of

coronatine on G6PDH happens post-transcriptionally. DS is the key enzyme of the shikimic acid pathway (Tran et al. 2011). Shikimic acid was the raw material to participate in cephalotaxine synthesis (Talapatra and Talapatra 2015). DS activity of suspension cells treated with coronatine and MJ was all ascended (Fig. 6) after 24 h of adding them, which may be the critical time for DS activity ascent.

Transcriptome analysis was a useful approach to explore the synthesis pathway of plant secondary metabolite. The first step of transcriptome analysis was how to obtain enough DEGs among different treatment cells. Then some information of key enzyme genes involved in the biosynthesis pathway of objective secondary metabolite would be discovered through analyzing those DEGs. Elicitors such as coronatine and MJ can disturb the normal metabolism of plant and result in many DEGs. Table 4 displayed that MJ treated cells had more DEGs than in coronatine treated cells, which possibly caused more non-objected secondary metabolites besides cephalotaxine in MJ treated cells. The objective product, cephalotaxine yield in MJ-treated cells was lower than that in coronatine treated cells, which may be contributed to that more substance and energy flux to other secondary metabolites but not to cephalotaxine in MJ-treated cells.

Transcriptome analysis (Table 5) and pathway enrichment analysis (Fig. 7) showed that coronatine-regulated enzymes were mainly involved in glucide metabolism (e.g., hexokinase), shikimic acid (e.g., DS), and phenylpropane pathways (e.g., PAL). Most of these enzymes were closely related to erythrose-4-phosphate synthesis. Judging by the present results, the expression levels of cytochrome P450, DS, hexokinase, phenylalanine ammonialyase (PAL), chalcone isomerase, and 3-phosphate glyceraldehyde dehydrogenase, etc. were promoted in coronatine-treated cells (Table 5). At the same time, gene expression levels of enzymes including alcohol dehydrogenase, sorbitol dehydrogenase, Caffeoyl-CoA *O*-methyltransferase up-regulated. Among the up-regulated enzyme genes, PAL was a key enzyme involved in plants' response to environmental stress (Dixon and Paiva 1995; Ge et al. 2019). The increasing DS transcription level provided more precursors for the production of cephalotaxine (Suzuki et al. 1996). Hexokinase was a key enzyme involved in glucide metabolism. As a sensor of plant sugar metabolism, up-regulation of the hexokinase gene was a benefit to improve glucose metabolism, and provided sufficient precursors for the synthesis of cephalotaxine (Jang et al. 1997).

Combined with the above analysis, gene expression of G6PDH and DS in the COR treatment group were improved, which indicated that G6PDH and DS were beneficial to product accumulation (Wei et al. 2019). According to the present results, Coronatine up-regulated the transcription levels of Hexokinase and DS, thereby provided more precursors for cephalotaxine synthesis, finally increased the metabolic flow of the cephalotaxine synthesis pathway.

Conclusion

In this study, coronatine had lower inhibition on the growth which is beneficial for *C. mannii* cells to produce cephalotaxine. The optimal addition of coronatine was 1.0 µmol/L, and the highest yield of cephalotaxine was 6.75 mg/L, which was twice that of the control group (3.14 mg/L). The optimal addition time was 15 d after inoculation. According to the results, the elicitation effect of lower concentration coronatine was better than the higher concentration. Lower concentration coronatine improved the DS and G6PDH activity, high concentration coronatine (2.0 µmol/L) caused cell death and even the autolysis of cells, it led to a negative effect on cell viability and product formation.

Transcriptome analysis showed that phenylalanine ammonialyase, DS, chalcone isomerase, laurate oxidase, hexokinase, and sorbitol dehydrogenase might play an important role in the biosynthesis pathway of cephalotaxine in the suspension cells of *C. mannii*. The mechanisms of these enzymes on the synthesis of cephalotaxine should be further explored. The cephalotaxine biosynthesis was mainly involved in glucide metabolism, shikimic and phenylpropane pathways, and tyrosine synthesis pathway.

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Author contributions This research was conceived and designed by LW and YL. The specific experiments were conducted by LW and ZW. The data analysis and writing were accompanied by LW and QZ. The manuscript was finally revised by YL.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval No human participants and /or animals are involved in this work.

Consent for publication All the authors agree with the publication of the article.

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