

# Effect of elicitors, precursors and metabolic inhibitors on paclitaxel production by *Taxus cuspidata* cell culture

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Received: 27 August 2014 / Accepted: 24 November 2014 / Published online: 3 June 2016  
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**Abstract** In this study, using *Taxus cuspidata* as a raw material, we obtained stable high-yielding cell lines by subculturing and quantified paclitaxel content using ultrasonic extraction combined with TLC–UV spectrophotometry. In single factor and multiple factors tests to optimize design and study the effects of elicitors, precursors, and metabolic inhibitors on paclitaxel production by *Taxus cuspidata* cells, paclitaxel production reached 4.32 mg/L when 100 μmol/L methyl jasmonate, 20 mg/L salicylic acid, 400 mg/L phenylalanine and 2 mg/L gibberellin (GA<sub>3</sub>) were added to the culture medium of suspension cells. When adding metabolic adjustment factors on the 7th day of culture, extra- and intracellular paclitaxel production was the highest at 4.855 mg/L, paclitaxel release rate was 10.48 %, fresh mass and paclitaxel production of cell increased, respectively, by 6.08 and 11.57 %. By

controlling the anabolism of paclitaxel, paclitaxel yield was significantly improved.

**Keywords** Elicitor · Precursor · Metabolic inhibitor · Paclitaxel · Cell culture · *Taxus cuspidata*

## Introduction

*Taxus cuspidata* produces the terpenoid paclitaxel (or taxol). This best-selling anticancer drug far surpasses many other antitumor drugs in annual global sales (Kwon et al. 1998). Paclitaxel, known as “plants gold”, inhibits the proliferation of tumor cells and has distinct therapeutic effects in the prevention and treatment of various cancers. However, *Taxus* is a slow-growing plant with extremely low content of paclitaxel that cannot meet current market demand (Liu and Ye 1999; Li 2009; Zhou et al. 2002). Because *Taxus cuspidata* is under state protection, it is imperative that we use modern technology to obtain paclitaxel through different pathways on the premise of protecting *Taxus*. Cell culture production of paclitaxel has many advantages and is becoming more and more popular with researchers (Tang and Zhong 2006; Gao et al. 2011; Seki et al. 1997; Zhong 2002). Most research has focused on basic theory (Zhai et al. 2009; Zhai and Fu 2010; Yu et al. 2013; Li et al. 2009; Zhao and Yang 2014; Kan et al. 2009); studies on the development of technology for efficient production of paclitaxel efficiently has been limited. In particular, work on systems for large-scale industrial production has not been reported. In this paper, *Taxus cuspidata* was used as a raw material. By optimization of the detection technology, callus induction, and screening for high-yield cell lines, we established a cell suspension system to improve paclitaxel production by adding and

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Project funding: This work was supported by development plan project during “the 12th Five Year Plan” Nation Science and Technology in rural area (No. 2012AA10A506-04 and No. 2013AA103005-04), Changchun City science and technology development program (No. 2014174) and Changchun City science and technology support program (No. 2014NK002).

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Corresponding editor: Chai Ruihai

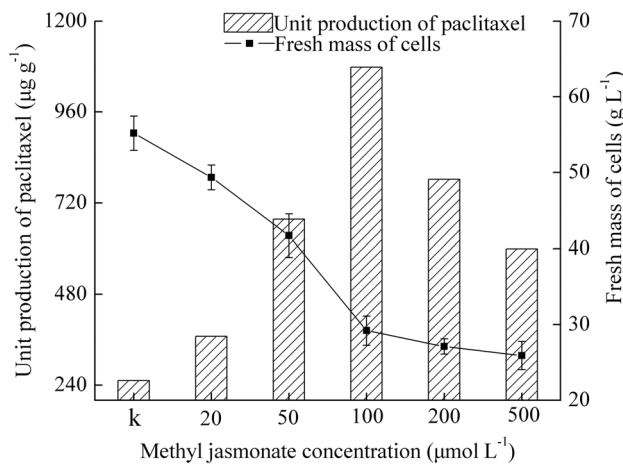
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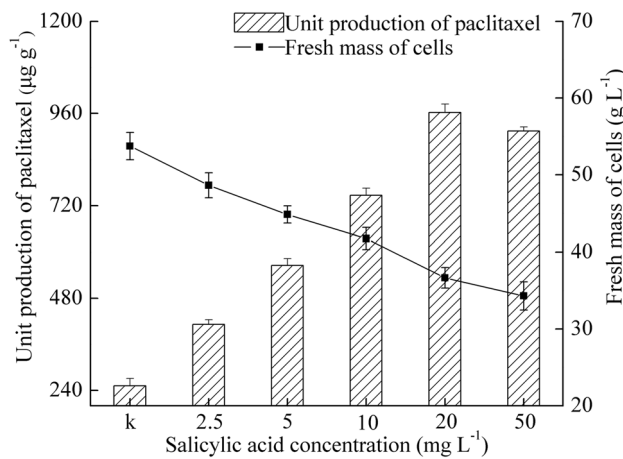
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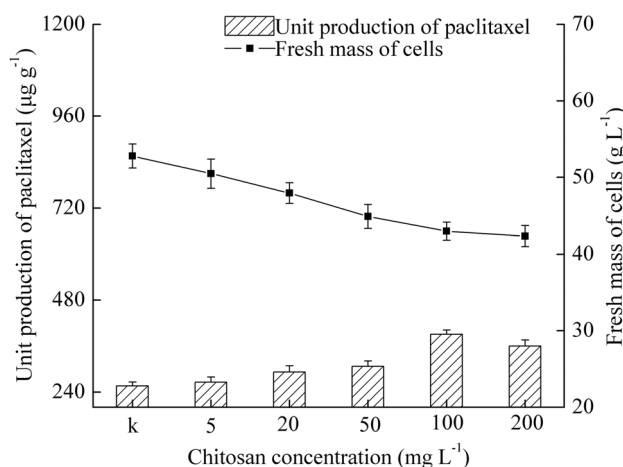
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**Fig. 1** The effect of methyl jasmonate on cell growth and paclitaxel production



**Fig. 2** The effect of salicylic acid on cell growth and paclitaxel production



**Fig. 3** The effect of chitosan on cell growth and paclitaxel production

adjusting metabolic factors. This study established a good foundation for large-scale, commercial, industrial production of paclitaxel using *Taxus cuspidata*, providing technical support for reasonable and efficient use of wild medicinal plants of Changbai Mountain, which has significant, important, far-reaching social benefits.

## Materials and methods

### Materials

*Taxus cuspidata* (source in Changbai Mountain) callus was obtained through repeated merit-based screening during subculture.

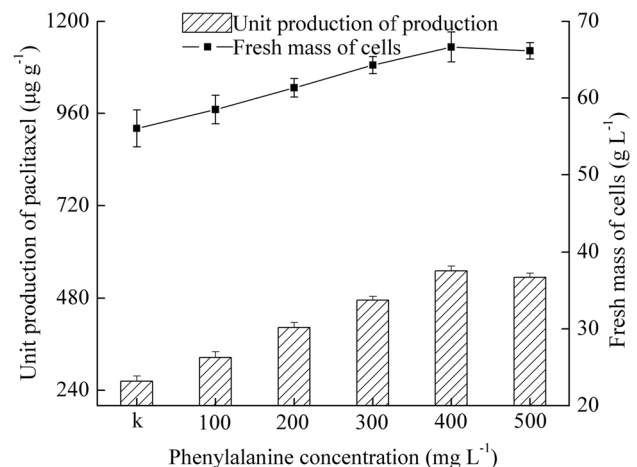
### Test methods

#### Suspension culture

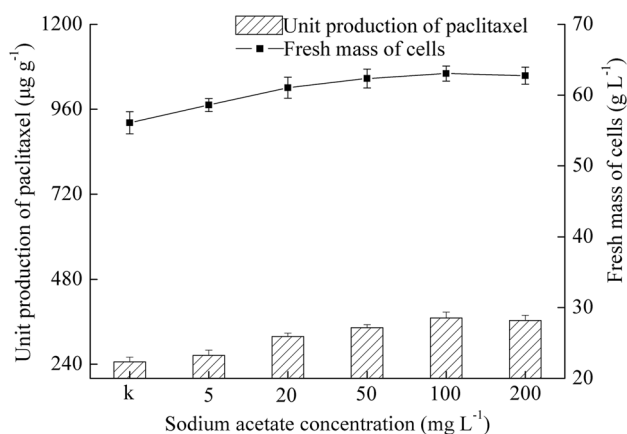
Suspension culture medium: improved B<sub>5</sub> liquid medium, containing basic B<sub>5</sub> liquid medium (Zhang 2009), 20 g/L sucrose, 2 mg/L glycine, 7.5 g/L citric acid, 2.0 mg/LNAA (naphthylacetic acid), 0.5 mg/L 6-BA (6- benzyl adenine). Culture conditions: temperature:  $25 \pm 1$  °C, speed: 120 r/min, dark. Oscillation culture in a constant temperature oscillation incubator (Shanghai yiheng Scientific Instrument Co., Ltd., Shanghai).

#### Extraction of paclitaxel

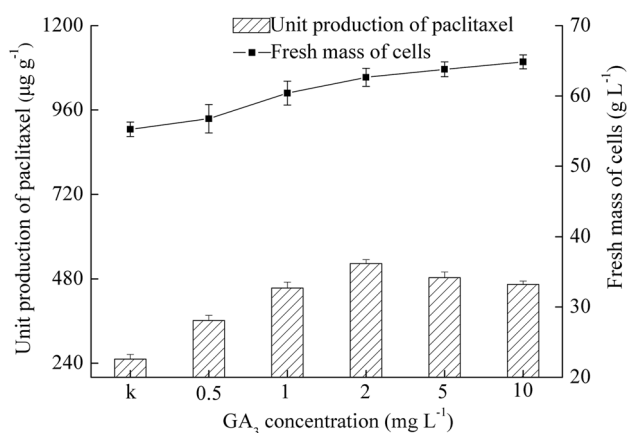
- (1) *Extraction of intracellular paclitaxel* Cells plus liquid suspension culture medium were centrifuged using LD 4-2A low speed centrifuge (Beijing Reble centrifuge Co. Ltd., Beijing) at 5000 r/min for



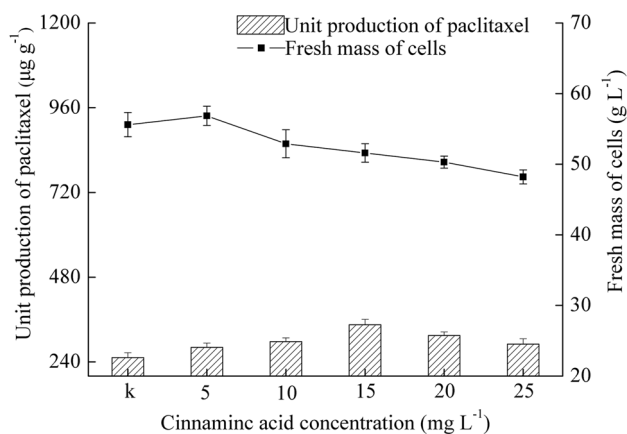
**Fig. 4** The effect of phenylalanine on cell growth and paclitaxel production



**Fig. 5** The effect of sodium acetate on cell growth and paclitaxel production



**Fig. 6** The effect of GA<sub>3</sub> on cell growth and paclitaxel production



**Fig. 7** The effect of cinnamic acid on cell growth and paclitaxel production

20 min. The resultant supernatant containing *Taxus* cells was incubated at 40 °C, dried to constant mass and ground to a powder with a mortar; there was

100 mg of the cell powder added to 2 mL of methanol and sonicated for 1 h. The mixture was filtered through the filter, repeating this filtration 3–5 times.

- (2) *Extraction of extracellular paclitaxel* (Qiao et al. 2009; Kan et al. 2009; He et al. 2010) A quantity of cell suspension culture medium was centrifuged using LD 4-2A low speed centrifuge (Beijing Reble centrifuge Co. Ltd., Beijing) at 5000 r/min for 20 min, and 10 mL of the resultant supernatant placed in a test tube, adjusted to pH 7.0 using 1 N NaOH or 1 N HCl and 3 mL of ethyl acetate added. The mixture was thoroughly shaken and allowed to separate through standing, before collection of the ethyl acetate layer. The extraction step was repeated twice, then the 9 mL of ethyl acetate from the three extractions was dried under reduced pressure and dissolved in 5 mL ethanol.

#### Quantification of paclitaxel content

The crude paclitaxel extract was separated with thin layer chromatography. At the  $R_f$  expected for paclitaxel, the silicone was scraped off and placed in 5 mL of ethanol, centrifuged for 15 min at 16,000 r/min to extract the paclitaxel. Absorbance of the sample was measured with a TU-1810 ultraviolet visible spectrophotometer (Beijing Purkinje General Instrument Co. Ltd, Beijing) using ethanol as a blank at the maximum absorption peak (228 nm). Paclitaxel content was obtained by reference to a paclitaxel standard curve.

#### Single factor experiments

On the first 7 days of culture of the cell suspension, different factors and concentrations of elicitors, precursors, or metabolic inhibitors were added to the medium. The corresponding concentration of each additive is shown below.

Methyl jasmonate ( $\mu\text{mol/L}$ ): 20, 50, 100, 200, 500; salicylic acid (mg/L): 2.5, 5, 10, 20, 50; chitosan (mg/L): 5, 20, 50, 100, 200; phenylalanine (mg/L): 100, 200, 300, 400, 500; sodium acetate (mg/L): 5, 20, 50, 100, 200; GA<sub>3</sub> (mg/L): 0.5, 1, 2, 5, 10; cinnamic acid (mg/L): 5, 10, 15, 20, 25.

Suspension cells were harvested after culturing for 28 days. Cell mass and unit production of intracellular paclitaxel were measured.

#### Multi-factor experiments

In the single factor experiments, several substances significantly increased paclitaxel production by the

**Table 1** Test results analysis of the quadratic general rotary combination

Factors test numbers	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	X <sub>4</sub>	Y (mg/L)
1	1	1	1	1	2.84
2	1	1	1	-1	2.88
3	1	1	-1	1	2.6
4	1	1	-1	-1	2.64
5	1	-1	1	1	2.68
6	1	-1	1	-1	2.76
7	1	-1	-1	1	2.44
8	1	-1	-1	-1	2.48
9	-1	1	1	1	3
10	-1	1	1	-1	3.08
11	-1	1	-1	1	2.92
12	-1	1	-1	-1	2.92
13	-1	-1	1	1	2.88
14	-1	-1	1	-1	3
15	-1	-1	-1	1	2.68
16	-1	-1	-1	-1	2.84
17	-2	0	0	0	2.92
18	2	0	0	0	2.48
19	0	-2	0	0	2.92
20	0	2	0	0	3.16
21	0	0	-2	0	3.24
22	0	0	2	0	3.64
23	0	0	0	-2	2.08
24	0	0	0	2	1.96
25	0	0	0	0	4.28
26	0	0	0	0	4.36
27	0	0	0	0	4.32
28	0	0	0	0	4.28
29	0	0	0	0	4.32
30	0	0	0	0	4.32
31	0	0	0	0	4.36

suspension culture. A quadratic general rotary combined design was used.

## Results and discussion

### Effect of elicitors, precursors and metabolic inhibitors on cell growth and paclitaxel production

#### *Effect of elicitor on cell growth and paclitaxel production*

As seen from Figs. 1, 2 and 3, as the elicitor concentration increased, the fresh cell mass of *Taxus cuspidata* showed a downward trend, that is, cell growth was inhibited; unit production of paclitaxel increased initially before reaching a maximum and then decreased. This result is due to the

elicitor stimulating the plant defense system, thus promoting greater paclitaxel synthesis (Gao et al. 2011; Laskaris et al. 1999; Raskin 1992; Mei et al. 2001). However, the elicitor was somewhat toxic as shown by the gradual decrease in fresh mass with increasing elicitor concentration.

On the basis of the fresh mass of cells and the highest paclitaxel yield per unit, the optimal concentrations of elicitors were: 100  $\mu\text{mol/L}$  methyl jasmonate, 20 mg/L salicylic acid, 100 mg/L chitosan with the yield of paclitaxel per unit of the respective elicitors, respectively, 4.28 times, 3.82 times, and 1.34 times the yield of the control group.

#### *Effect of precursor on cell growth and paclitaxel production*

As shown in Figs. 4 and 5, as the precursor concentration increased, the fresh mass of cells and unit production of paclitaxel increased slowly at first, then plateaued. Therefore, addition of the correct kind and concentration of precursors can increase the fresh mass of cells in suspension culture and improve the unit yield of paclitaxel. The optimal concentration of phenylalanine was 400 and 100 mg/L for sodium acetate; the yield of paclitaxel per unit was, respectively, 2.09 times and 1.51 times the yield of the control group.

#### *The effect of metabolic inhibitor on cell growth and paclitaxel production*

As seen in Figs. 6 and 7, the different metabolic inhibitors had different effects on the fresh mass of the cells in suspension culture. Increases in the GA<sub>3</sub> concentration promoted cell growth, but increases in cinnamic acid inhibited growth. For both compounds, as their concentration was increased, unit production of paclitaxel increased at first, then reached a maximum, and then decreased slightly. This pattern was due to inhibition of branching pathways unrelated to synthesis of paclitaxel, so the reaction tended to the direction of paclitaxel synthesis. Gibberellin with auxin promotes cell division and can improve cell fresh mass; however, cinnamic acid has somewhat toxic effect on cell growth (Li et al. 2009). Optimal concentration of gibberellin and cinnamic acid was respectively 2 and 15 mg/L, the yield of paclitaxel per unit was, respectively, 1.37 times and 2.08 times compared with that of the control group.

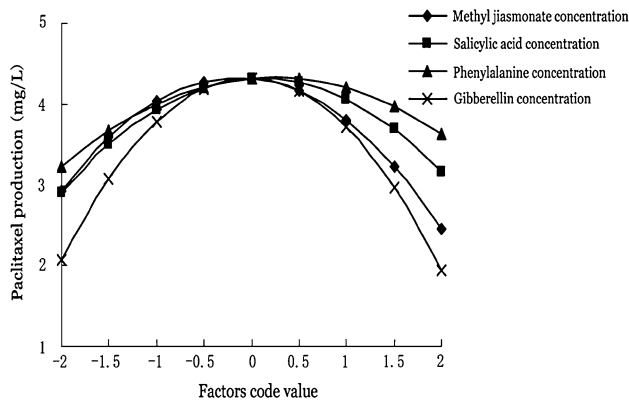
### Optimization of paclitaxel synthesis by *Taxus cuspidata* cells

#### *Design and results*

On the basis of the results of the single factor tests, the four compounds that yielded a relatively large increase in

**Table 2** Analysis of variance of the test results

Source of variation	Sums of squares	DOF	Mean squares	F ratio	p value
$X_1$	0.2592	1	0.2592	324	0.0001
$X_2$	0.08	1	0.08	100	0.0001
$X_3$	0.18	1	0.18	225	0.0001
$X_4$	0.02	1	0.02	25	0.0001
$X_1^2$	3.5468	1	3.5468	4433.532	0.0001
$X_2^2$	2.2191	1	2.2191	2773.862	0.0001
$X_3^2$	1.0538	1	1.0538	1317.266	0.0001
$X_4^2$	7.132	1	7.132	8915.021	0.0001
$X_1X_2$	0.0003	1	0.0003	0.375	0.5489
$X_1X_3$	0.0075	1	0.0075	9.375	0.0075
$X_1X_4$	0.0012	1	0.0012	1.5	0.2384
$X_2X_3$	0.0012	1	0.0012	1.5	0.2384
$X_2X_4$	0.0027	1	0.0027	3.375	0.0848
$X_3X_4$	0.0003	1	0.0003	0.375	0.5489
Return	15.5387	14	1.1099	$F_2 = 1387.382$	0.0001
Surplus	0.0128	16	0.0008		
Lack of fit	0.0064	10	0.0006	$F_1 = 0.600$	0.792
Error	0.0064	6	0.0011		
Sum	15.5515	30			

**Fig. 8** Analysis diagram of single factor effect

production of paclitaxel by cells were selected for further tests: methyl jasmonate, salicylic acid, phenylalanine and GA<sub>3</sub>. Interactions among these four substances were studied by adopting a quadratic general rotary combined design. Analysis of the results is shown in Table 1.

Statistical analysis software DPS v9.50 (Home Page, China) was used for processing data from Table 1; the regression equation was as follows:

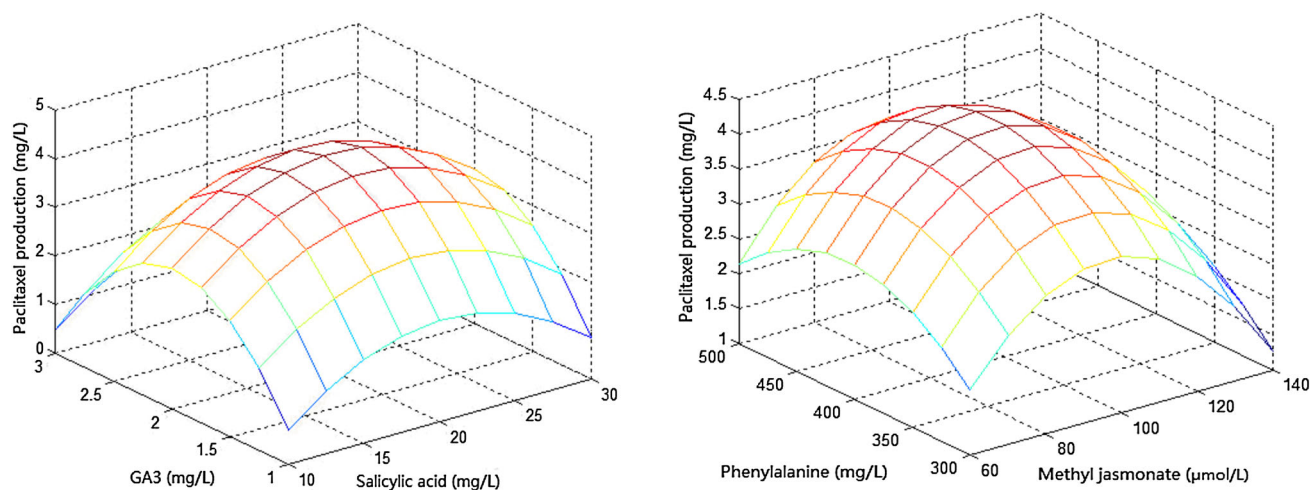
$$Y = 4.32000 - 0.12000X_1 + 0.06667X_2 + 0.10000X_3 - 0.03333X_4 - 0.40667X_1^2 - 0.32167X_2^2 - 0.22167X_3^2 - 0.57667X_4^2 + 0.00500X_1X_2 + 0.02500X_1X_3 + 0.01000X_1X_4 - 0.01000X_2X_3 + 0.01500X_2X_4 - 0.00500X_3X_4$$

As can be seen from Table 2, the regression equation is highly significant, and lack of fit is not significant, indicating that the equation fit well with the actual situation. After excluding items that were not significant at  $\alpha = 0.10$ , the simplified regression equation was as follows:

$$Y = 4.32000 - 0.12000X_1 + 0.06667X_2 + 0.10000X_3 - 0.03333X_4 - 0.40667X_1^2 - 0.32167X_2^2 - 0.22167X_3^2 - 0.57667X_4^2 + 0.02500X_1X_3 + 0.01500X_2X_4$$

#### The effect of single factor analysis

When three of the four factors were fixed at the zero level, a single-factor model was used to describe the effect of changes in a single factor on paclitaxel production. The single factor effect analysis is shown in Fig. 8.



**Fig. 9** Interaction effect diagram

**Table 3** Frequency distribution optimization of each variable value

Factor level	$X_1$		$X_2$		$X_3$		$X_4$	
	Times	Frequency	Times	Frequency	Times	Frequency	Times	Frequency
-2	0	0	0	0	1	0.0169	0	0
-1	17	0.2881	13	0.2203	13	0.2203	14	0.2373
0	30	0.5085	27	0.4576	22	0.3729	33	0.5593
1	12	0.2034	18	0.3051	18	0.3051	12	0.2034
2	0	0	1	0.0169	5	0.0847	0	0
Weighed average number	-0.085		0.119		0.22		-0.034	
SD	0.091		0.099		0.122		0.086	
95 % of the distribution range	-0.262 to 0.093		-0.076 to 0.313		-0.020 to 0.460		-0.203 to 0.135	

As can be seen from Fig. 8, univariate effects of four factors showed a parabolic shape. Because the coefficients of the quadratic terms of four factors are negative, the parabola opens downward, indicating that there were maximums in this test. The total yield of intracellular paclitaxel increased initially and then decreased as the concentration of the four additives increased.

#### Interaction analysis

As can be seen from the regression equation and Table 2, the interactions in Fig. 9 among  $X_1$  (methyl jasmonate),  $X_3$  (phenylalanine),  $X_2$  (salicylic acid) and  $X_4$  ( $GA_3$ ) are significant. The point values on the interaction effect diagram agreed with the results of the single factor effect analysis, illustrating that the test and the equation were accurate.

#### Optimization of extraction and validation process

As can be seen in Table 3, when the values of  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$  were -0.262 to -0.093, -0.076 to -0.313, -0.020 to -0.460, -0.203 to -0.135, respectively, intracellular paclitaxel production may be more than 3.14 mg/L with a 95 % possibility. The actual concentrations of methyl jasmonate, salicylic acid, phenylalanine and  $GA_3$  were 94.76–101.86  $\mu\text{mol/L}$ , 19.62–21.565 mg/L, 399–423 mg/L and 1.8985–2.0675 mg/L respectively. The total yield of intracellular paclitaxel was more than 3.14 mg/L in ten repeated tests in the same conditions, confirming that the above analysis was accurate.

$Y_{\text{max}}$ , the maximum of intracellular paclitaxel yield, was 4.32 mg/L when the values of  $X$  were between -2 and 2. As mentioned above was the best solution of regression model. The highest value for each combination of factors is shown

below. When the concentration of methyl jasmonate is 100  $\mu\text{mol/L}$ , salicylic acid is 20  $\text{mg/L}$ , phenylalanine is 400  $\text{mg/L}$  and  $\text{GA}_3$  is 2  $\text{mg/L}$ , the total production which depends on suspension cell biomass of *Taxus cuspidata* and unit production of paclitaxel reaches the maximum of 4.32  $\text{mg/L}$ .

## Conclusions

By single factor test studying effects of several elicitors, precursors and metabolic inhibitors on paclitaxel production by *Taxus cuspidata* cell culture, methyl jasmonate, salicylic acid, phenylalanine and  $\text{GA}_3$  were screened out for their relatively large influence.

The test with the factors of methyl jasmonate ( $X_1$ ), salicylic acid ( $X_2$ ), phenylalanine ( $X_3$ ), and  $\text{GA}_3$  ( $X_4$ ) was conducted by adopting the quadratic general rotary combined design. The regression equation was simplified as:

$$Y = 4.32000 - 0.12000X_1 + 0.06667X_2 + 0.10000X_3 - 0.03333X_4 - 0.40667X_1^2 - 0.32167X_2^2 - 0.22167X_3^2 - 0.57667X_4^2 + 0.02500X_1X_3 + 0.01500X_2X_4$$

The optimal combination of additive concentration was determined for methyl jasmonate 100  $\mu\text{mol/L}$ , salicylic acid 20  $\text{mg/L}$ , phenylalanine 400  $\text{mg/L}$  and  $\text{GA}_3$  2  $\text{mg/L}$ .

When adding adjustment factor on the 7th day of suspension cells growth, extracellular and intracellular paclitaxel production was the highest at 4.855  $\text{mg/L}$ , paclitaxel release rate was 10.48 %, fresh mass and paclitaxel production of cell increased respectively by 6.08 and 11.57 %.

**Acknowledgments** We thank College of Biological and Agricultural Engineering, Key Laboratory of Bionic Engineering (Ministry of Education) as well as College of Food Science and Engineering in Jilin University for support.

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