The Effect of The Combination of Precursor and Elicitors Enhance the Taxol Accumulation in Red Pine Cell Cultivation

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Abstract: **The material introduced into the callus culture was the in vitro cloned red pine stem and leaves. Callus obtained through culture from leaves and stems were included in the study on proliferation on agar and liquid media. The suspension obtained after 45 days of culture was determined for biomass, used in proliferation culture, and studied taxol accumulation.**

Media for taxus cell cloning was MS supplemented with 3 mg/l 2.4D, 3 mg/l NAA, 0.5 mg/l kinetin, 0.1 mg/l BA, 10% CW. Selections were carried out through 8 steps in the year of 2010 with interval cultivation time of 45 days/each step. It's could improve the taxol accumulation via cell suspension cultures sourced from leaves 170.1 mg/gDW and stems 27.3 mg/gDW. Picloram was not effect on taxol accumulation.

On the basic media MS supplemented with 3 mg/l 2.4D, 3 mg/l NAA, 0.5 mg/l kinetin, 0.1 mg/l BA, 10% CW supplemented with precursor of 15mg/l phenyl alanine (PA) effects on the percentage of FW/DW (fresh weight/dried weight) was 9.815 and the taxol accumulation was 0.778%. The effects of elicitors supplemented to media with 10 mg/l methyl jasmonate (MJ) having 7.183 FW/DW and 0.273% taxol, 100 mg/l salysilic acid (SA) having 10.12 FW/DW and 0.094% taxol, 50mg/l chitosan having 10.09 FW/DW and 0.119% taxol, 5mg/l oligo-chitosan having 9.090 FW/DW and 0.778% taxol.

The effects of the combination of precursor and elicitors (15 mg/l phenyl alanine, 10 mg/l methyl jasmonate, 5 mg/l O-chitosan, 100 mg/l salysilic acid) enhance the taxol accumulation to 1.003% in comparison of separately as PA having 0.114% taxol, PA+Ochi having 0.542% taxol, PA+MJ+Ochi having 0.564%. Cell cloning from leaves (1.701%) had the taxol accumulation more than from stem (0.273%).

Keywords: **Red pine, cell cloning, cell suspension, precursor, elicitor**

I. INTRODUCTION

Red pine (Taxus sp.) is the only source of raw materials currently providing bark and leaves used for taxol extraction. However, the main limitation is that red pine trees grow slowly, have low taxol content (only 0.01% dry bark) and 2-3 red pine trees > 50 years old can only produce 1 g taxol to treat one disease (Gibson *et al.*, 1993), and need >1000 kg taxol/year to treat all kinds of cancer, the selling price of 25 mg taxol is 685 USD.

Cell culture is a viable technique to produce taxol under stable and time-continuous production conditions (Zhong *et al.*, 1997). An important barrier to overcome in culture techniques is the selection of cell lines that are genetically, biochemically, and physiologically appropriate (Jaziri *et al.*, 1996). With traditional cell culture techniques, we only get average results due to heterogeneous cell populations (Yanpaisan *et al.*, 1999). To achieve this degree of cloning, the key technique is cloning (Ma *et al.*, 2003).

Culture conditions, and precursors and elicitors were used to enhance paclitaxel production efficiency. Phenylalanine and its precursors in the paclitaxel biosynthetic chain improved paclitaxel accumulation in callus culture and cuspidata T cells (Fett-Neto *et al.*, 1994). Elicitors are introduced into the culture medium to increase the content of secondary substances produced (Yu *et al.*, 2002). Elicitors can be glucan polymers, glycoproteins, small molecule organic acids, or fungal cells; or unfavorable biological conditions (abiotics) such as UV rays, heavy metal salts and other chemicals. Yukimune *et al.* (1996) noted that methyl jasmonate (MJA) treatment greatly improved the production cost and taxol recovery efficiency. Effects of elicitor-like substances such as arachidonic acid (Srinivasan *et al.*, 1996), silver ion (Zhang *et al.*, 2000), chitosan (Linden and Phisalaphong 2000), La^{3+} ion (Wu *et al.*, 2001) on paclitaxel production was studied, salycylic acid (SA) improved the biomass and accumulation of paclitaxel compared with only the addition of elicitor of mushroom extract.

In this paper, we study the possibility of selecting a red pine (Taxus sp.) cell line with high proliferative capacity and study the influence of precursor and elicitors on the ability to accumulate taxol through cell suspension culture.

II. MATERIALS AND METHOD

Red pine cell culture

Materials

The material introduced into the callus cell culture was the in vitro cloned red pine stem and leaves. Callus cells obtained through culture from leaves and stems were included in the study on proliferation on agar and liquid media. The suspension obtained after 45 days of culture was determined for biomass, used in proliferation culture, and studied taxol accumulation.

Cell density: counted by a red blood cell counting chamber (with a Thoma counting frame structure, including 25 large cells and 16 small cells in each large cell, the area of each small cell is 1/400mm2, the height of each cell is 0.1 mm in a drop of solution, then calculated in 1 ml of solution with a dilution of 10-1 with the formula: Number of cells/ml sample = [a x 4000 x 1000]/H. With: a: average number of cells in a microfield area (small cell) and H: dilution factor.

The basic culture medium in the study on proliferation and proliferation of callus cells on MS liquid medium, supplemented with CW (10%), B1 (5 mg/l), Glycin (5 mg/l), sucrose (30 g/l), 3 mg/l 2.4D (2.4-dichlorophenoxy acetic acid), 3 mg/l NAA (α-naphthalene acetic acid), 0.1 mg/l BA (6-benzyl aminopurin), 0.5 mg/l kinetin (furfuryl aminopurine), picloram. These were purchased from Sigma Co.

Precursor used: phenyl alanine (PA).

Elicitors used: methyl jasmonate (MJ), chitosan (Chi), oligo-chitosan (Ochi), salysilic acid (SA).

Culture conditions: the medium was sterile at 121 ^{o}C, 1 at, for 25 min. Room temperature 28+1 oC, light intensity 33.3 µmol/m2/s, lighting time 8 hours/day.

Cell culture on a shaker with a shaking speed of 80 rpm

Method

The experiment was arranged according to RBD, each treatment was repeated 3 times. Each replicate was cultured in three 300 ml conical flasks, with a culture volume of 50ml/300ml flask. The data were processed using statistical software MSTATC.

Extraction and quantification of taxol stem and leaf cell suspensions

Procedure for extracting dried leaf samples

Weighing 5 g of dried leaf powder was solvent with 100 ml of EtOAc (ethyl acetate). Then, the compose was ultrasonic surface (3 times, 10 minutes each time). Rotate and collect the matter (this method is used for tissue and cell samples).

Sample preparation for HPLC analysis

Matter weight 0.691 g was dissolved in 20 ml MeOH (label T3).

Analysis and quantification of taxol by HPLC

HPLC running conditions for moblie phase by using detector DAD (diot array detector); with absorption spectrum: 210-230 nm; column: C1; flow rate: 0.5 ml/min; sample injection volume: 10 µl.

HPLC running conditions

	H2O	MeOH	AcN
0-3 min	30	70	
3 min	50	50	
15 min	10	90	
25-30 min	10	40	50
35 min	30	70	

MeOH: Methanol; AcN: Acetonitrile

III. RESULTS

Table 1: Selection of fast-growing cell lines

Sample	1	2	3	4	5	6	7	8
Leaf	4.13	4.27	4.83	5.10	5.80	6.82	6.91	7.05
CV%	2.06	2.13	2.41	2.55	2.90	3.41	3.45	3.52
Stem	4.51	5.47	6.22	7.11	7.21	8.02	8.54	9.62
CV%	2.25	2.73	3.11	3.55	3.60	4.01	4.27	4.81

Table 2: Effect of picloram on the growth of red pine cell suspension

Pichloram (mg/l)	Fresh Weight (mg/50ml)	Dry Weight (mg/50ml)	DW/FW
0.0	3.61d	0.24d	6.648
0.1	5.34c	0.53b	9.925
0.5	5.40c	0.58b	10.74
1.0	6.79b	0.65a	9.572
1.5	5.46c	0.53b	9.706
2.0	9.27a	0.33c	3.559

Table 3: Effect of methyl jasmonate on the ability to accumulate taxol

Methyl jasmonate (mg/l)	Fresh Weight (mg/50ml)	Dry Weight (mg/50ml)	DW/FW	HPLC (ppm)	Taxol (mg/g dry)	Rate of Taxol (%)
0	3.26c	0.28c	8.588	21.308d	11.4c	0.114c
10	3.48b	0.25c	7.183	43.835a	26.3a	0.263a
20	5.72a	0.55a	9.615	42.485b	11.5c	0.115c
30	3.27c	0.33b	10.09	26.309c	11.9b	0.119b

Table 4: Effect of salicylic acid on the ability to accumulate taxol

Salicylic acid (mg/l)	Fresh Weight (mg/50ml)	Dry Weight (mg/50ml)	DW/FW	HPLC (ppm)	Taxol (mg/g dry)	Rate of Taxol (%)
0	10.9a	1.29a	11.83	22.791f	2.6f	0.026f
10	8.21c	0.95d	11.57	23.165e	3.6e	0.036e
50	9.19b	1.03c	11.20	43.233a	6.2b	0.062b
100	5.63e	0.57f	10.12	36.098b	9.4a	0.094a
150	7.87d	0.89e	11.30	34.767c	5.8c	0.058c
200	9.62b	1.13b	11.74	31.669d	4.2d	0.042d

Table 5: Effect of chitosan on the ability to accumulate taxol

Chitosan (mg/l)	Fresh Weight (mg/50ml)	Dry Weight (mg/50ml)	DW/FW	HPLC (ppm)	Taxol (mg/g dry)	Rate of Taxol (%)
0	9.10b	1.07b	11.75	52.608a	7.3c	0.073c
50	3.27e	0.33e	10.09	26.309e	11.9a	0.119a
100	8.15c	0.96c	11.77	51.857b	8.1b	0.081b
150	7.11d	0.70d	9.845	36.715d	7.8c	0.078c
200	9.64a	1.40a	14.52	43.509c	4.6d	0.046d

Table 6: Effect of oligo-chitosan on the ability to accumulate taxol

Oligo-chitosan (mg/l)	Fresh Weight (mg/50ml)	Dry Weight (mg/50ml)	DW/FW	HPLC (ppm)	Taxol (mg/g dry)	Rate of Taxol (%)
0	7.62a	0.74a	9.711	108.124c	21.9d	0.219d
5	4.51c	0.41c	9.090	212.869a	77.8a	0.778a
10	4.95c	0.48c	9.696	186.482b	58.2b	0.582b
15	6.12b	0.61b	9.967	186.592b	45.8c	0.458c

Table 7: Effect of phenyl alanine on the ability to accumulate taxol

Phenyl alanine (mg/l)	Fresh Weight (mg/50ml)	Dry Weight (mg/50ml)	DW/FW	HPLC (ppm)	Taxol (mg/g dry)	Rate of Taxol (%)
0	8.22b	0.93b	11.31	36.556e	5.8d	0.058d
5	9.87a	1.02a	10.33	52.663c	4.5e	0.045e
10	7.64c	0.82c	10.73	67.541a	12.3b	0.123b
15	6.52d	0.64d	9.815	57.751b	13.5a	0.135a
20	9.71a	1.15a	11.84	50.410d	6.5c	0.065c

Table 8: Effect of precursors (15 mg/l phenyl alanine) and elicitors (10 mg/l methyl jasmonate, 5 mg/l O-chitosan, 100 mg/l salysilic acid) on taxol accumulation

PA (mg/l)	Fresh Weight (mg/50ml)	Dry Weight (mg/50ml)	DW/FW	HPLC (ppm)	Taxol (mg/g dry)	Rate of Taxol (%)
PA	8.54b	0.93b	10.88	70.927e	11.4e	0.114e
PA+MJ	7.62c	0.74e	9.711	108.124d	21.9c	0.219c

PA+SA	9.62a	1.12a	11.64	118.521d	15.8d	0.158d
PA+OChi	3.66d	0.32f	8.743	115.825d	54.2b	0.542b
PA+MJ+SA	7.35c	0.75d	10.20	124.201c	24.8c	0.248c
PA+MJ+OChi	3.47d	0.36f	10.37	135.418b	56.4b	0.564b
PA+SA+OChi	7.89c	0.87c	11.02	131.732b	22.7c	0.227c
PA+MJ+Ochi+SA	3.52d	0.21g	5.96	140.448a	100.3a	1.003a

Table 9: Effect of culture sample on the ability to accumulate taxol (cell lines after 8 times of selection in 2010)

Sample	Fresh Weight (mg/50ml)	Dry Weight (mg/50ml)	DW/FW	HPLC (ppm)	Taxol (mg/g-dry)	Rate of Taxol (%)
Leaf	3.05	0.31	10.16	351.712	170.1	1.701
Stem	10.2	1.22	11.96	331.449	27.3	0.273

15 mg/l PA + 10 MJ + 100 mg/l SA + 5 mg/l O-chitosan

Table 10: Quantification of taxol accumulation through leaf and stem cell culture

Sample	Cell density (cell/ml)	Fresh Weight (g)	Dry Weight (g)	HPLC (ppm)
Sample 1	11.7×10^4	4.83	0.46	0
Sample 2	4.5×10^4	5.1	0.53	0
Sample 3	3.2×10^4	3.52	0.21	140.448
Sample 4	6.7×10^4	3.66	0.32	115.825
Sample 5	7.4×10^4	6.87	0.66	0.0
Sample 6	7.1×10^4	6.91	0.66	0.0
Sample 7	3.8×10^4	6.12	0.61	186.592
Sample 8	7.1×10^4	3.27	0.33	26.309
Sample 9	4.7×10^4	4.95	0.48	186.482
Sample 10	2.3×10^4	4.27	0.43	0.0
Sample 11	2.6×10^4	7.05	0.69	0.0
Sample 12	5.2×10^4	3.48	0.25	43.835
Sample 13	4×10^4	6.79	0.65	0.0
Sample 14	2.9×10^4	3.61	0.24	0.0
Sample 15	3.3×10^4	5.4	0.58	0.0
Sample 16	4.8×10^4	5.34	0.53	0.0
Sample 17	3.9×10^4	5.46	0.53	0.0
Sample 18	4×10^4	5.77	0.55	42.485
Sample 19	2.8×10^4	5.8	0.58	0.0
Sample 20	3.9×10^4	4.13	0.42	0.0
Sample 21	5.6×10^4	3.26	0.28	21.308
Sample 22	2.8×10^4	5.6	0.56	23.544
Sample 23	3×10^4	3.47	0.36	135.418
Sample 24	3.7×10^4	3.05	0.31	351.712
Sample 25	2.9×10^4	4.51	0.41	212.896
Sample 26	5.4×10^4	5.47	0.53	64.592
Sample 27	1.5×10^4	9.62	1.13	0.0
Sample 28	2×10^4	6.07	0.6	31.669
Sample 29	5×10^4	8.15	0.96	51.857

Sample 30	4.9×10^4	7.89	0.87	131.732
Sample 31	10.1×10^4	9.19	1.03	43.233
Sample 32	3.2×10^4	8.21	0.95	23.165
Sample 33	3.2×10^4	9.87	1.72	52.663
Sample 34	4.2×10^4	10.2	1.82	331.449
Sample 35	2.7×10^4	9.64	1.4	43.509
Sample 36	4.1×10^4	6.52	0.64	57.751
Sample 37	3.4×10^4	8.54	0.93	70.927
Sample 38	1.7×10^4	9.71	1.15	50.41
Sample 39	6.8×10^4	7.21	0.74	0.0
Sample 40	10.5×10^4	8.54	0.94	0.0
Sample 41	2×10^4	10.9	1.29	22.791
Sample 42	6×10^4	5.63	0.57	36.098
Sample 43	2.4×10^4	8.02	0.89	0.0
Sample 44	1.7×10^4	7.11	0.7	36.715
Sample 45	2.1×10^4	7.64	0.82	67.541
Sample 46	7.2×10^4	7.87	0.89	34.767
Sample 47	1.6×10^4	6.22	0.59	53.822
Sample 48	4.9×10^4	7.35	0.75	124.201
Sample 49	4.7×10^4	9.1	1.07	52.608
Sample 50	2.6×10^4	7.62	0.74	108.124
Sample 51	5.5×10^4	8.22	0.93	36.556
Sample 52	4.7×10^4	9.61	1.12	118.521

IV. DISCUSSION

Selection fast-growing cell lines

Research results were shown in Table 1 show that after 8 times of selection, the proliferation coefficient increases with the time of selective culture. One stem cell line has been selected with a proliferation coefficient of 4.81 and similar to that of the line selective leaf cell is 3.52.

Effect of picloram on the ability to accumulate taxol

Pichloram 1 mg/l affects the agglomeration process of 0.65 mg dry weight and DW/FW ratio 9.572 (Table 2). Among the treatments, none were used in taxol-accumulating cell cultures.

Effect of methyl jasmonate (MJ) on the ability to accumulate taxol

Adding to the culture medium methyl jasmonate 10 mg/l affected the ratio of DW/FW to low 7.183 (Table 3) but stimulated accumulation of high taxol 26.3 mg/g-dry with a high ratio of 26.3 mg/g-dry accumulated taxol content is 0.273%.

Effect of salicylic acid (SA) on the ability to accumulate taxol

The results in Table 4 showed that were added to the culture medium salysilic acid 100 mg/l affected the ratio of DW/FW as low as 10.12 but stimulated the accumulation of taxol 9.4 mg/g-dry with the ratio of taxol content was 0.094%.

Effect of Chitosan (Chi) on the ability to accumulate taxol

Culture medium was supplemented with chitosan 50 mg/l (Table 5) affected the ratio of DW/FW as low as 10.09 but stimulated accumulation of taxol 11.9 mg/g-dry with the ratio function; the amount of taxol accumulated was 0.119%.

Effect of oligo-chitosan (Ochi) on the ability to accumulate taxol

Results of the study in Table 6 show that O-chitosan 5 mg/l were added to the culture medium affects the ratio of DW/FW but stimulates the accumulation of taxol 77.8 mg/g-dry with the ratio of accumulated taxol content capacitor is 0.778%.

Effect of phenyl alanine (PA) on the ability to accumulate taxol

Adding phenyl alanine 15 mg/l (Table 7) to the culture medium give the ratio of DW/FW 9,815 but stimulates the accumulation of taxol 13.5 mg/g-dry with the taxol content ratio accumulation is 0.778%.

Effect of precursors and elicitors on the ability to accumulate taxol

the culture medium was supplemented with PA+MJ+Ochi+SA (Table 8) affected the ratio of DW/FW 5.96 but stimulated accumulation of taxol 100.3 mg/g-dry rate of taxol content is 1.003%.

Effect of samples on the ability to accumulate taxol

Leaf cell suspension culture had a low DW/FW ratio of 10.16, but stimulated the accumulation of taxol 170.1 mg/g-dry with the accumulation of taxol content of 1.701%. Similarly, leaf cell suspension culture had a low DW/FW ratio of 11.96 but stimulated the accumulation of taxol 27.3 mg/g-dry with the accumulation rate of taxol content of 0.273 % (Table 9)

Analysis and quantification of taxol

We use taxus cell suspension culture from leaves (samples 1-26) and from stem (samples 27-52). Analysis and quantification of taxus accumulation by HPLC. The highest value of taxol were 351.712 ppm on leaves cell (sample 24) and 331.449 ppm on stem cell (sample 34). But the data record were varied and not stable among the samples (Table 10)

V. CONCLUSIONS

Selected cell line was cultured on MS medium supplemented with 3 mg/l 2.4D, 3 mg/l NAA, 0.5 mg/l kinetin, 0.1 mg/l BA and 10% CW. The 8th selected cell line (45 days/one cycle of selection) was selected with the ability to improve taxol accumulation through culture of leaf cell suspension (taxol content 170.1 mg/g-dry) and stem (taxol content 27.3 mg/g-dry). Phenylalanine, a precursor, and methyl jasmonate, an elicitor, have effect on taxol accumulation. Adding to the MS culture medium with PA+MJ+Ochi+SA (precursor concentration of 15 mg/l phenyl alanine and elicitor 10 mg/l methyl jasmonate, 5 mg/l O-chitosan , 100 mg/l salysilic acid) affected the ability to accumulated taxol 100.3 mg/g-dry weight and ratio of taxol content to dry weight 1.003%

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