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The selection and stability analysis of stable and high Taxol-producing cell lines from *Taxus cuspidata*

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Abstract In this study to screen for stable, high Taxol-producing cell lines (CL5, CL12, and CL21) of *Taxus cuspidata*, stem tissues were used to induce calli, which were then subcultured nine times to establish suspension cell cultures. From 97 cell lines obtained from conditioned cultures, 10 cell lines with high Taxol content were selected. Stability analyses on solid and liquid B5 media were then used to obtain lines that stably produced high levels of Taxol. Fresh biomass and Taxol production of the ninth generation became stable. Taxol content of selected CL5, CL12, and CL21 samples was 0.0448, 0.0477, and 0.0428% of dry mass (DW), respectively. Proliferation of CL5, CL12 and CL21 was 346.3, 382.5, and 409.2%, respectively. From work over about 2 years, the three cell lines appear suitable for mass production of Taxol, promoting the industrialisation and commercial-scale production of Taxol using cell culture.

Keywords Paclitaxel · *Taxus cuspidata* · Taxol · Cell lines · Stability analysis

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Introduction

Taxol (paclitaxel), a potent anticancer drug is mainly extracted from roots, barks, stems, and needles of plants in the *Taxus* genus (Kwon et al. 1998; Pasquali et al. 2006; Bentebibel et al. 2005). However, plant sources are very scarce, and their Taxol content is very low (Bestoso et al. 2006). The Taxol content in callus tissue, however, is significantly higher than in stems, needles, and other plant parts (Expósito et al. 2009). Thus, plant tissue culture and cell culture are the most promising approaches to overcome the serious shortage of Taxol (Slichenmyer and Von-Hoff 1991; Kim et al. 2001; Zhong 2002; Tabata 2004). The low content and often unstable production of Taxol in cultured *Taxus* cells, however, has hampered large-scale production (Luo et al. 1999; Cusidó et al. 2002; Kim et al. 2004). Therefore, the primary task for cell culture is to develop an effective method to screen cell lines for stably high Taxol yields and growth rates. Of the many methods for generating high Taxol-producing cell lines, single-cell cloning has many advantages, such as large-scale, efficient screening for high stability (Seki et al. 1997; Ketchum et al. 2007). Many cell lines have been established from different species of the *Taxus* genus (Fettneto et al. 1992; Wickremesinhe and Arteca 1993; Malik et al. 2011; Frense 2007; Liu et al. 2016), such as the *Taxus baccata* callus line and its derived cell suspension culture described by Cusidó et al. (1999). Three cell lines from *Taxus globosa* were

selected and cultured by using methyl jasmonate as an elicitor, which apparently improved cell line growth (Barradasdermitz et al. 2010). However, detailed screening methods have rarely been mentioned. Also single-cell clones and conditioned cultures have rarely been used to select cell lines from plants of *Taxus cuspidata* or another *Taxus* species. Moreover, to obtain stable production of Taxol in *Taxus* cells, once high-yield stable cell lines have been selected, they must be screen further through repeated subculturing and stability analysis because cell lines may gradually produce less Taxol or lose the ability entirely.

In this work, we studied the effect of subculturing on the growth and Taxol production of *Taxus cuspidata* calli. Next, three methods for single-cell cloning were compared. After further culturing on conditioned media, lines were selected for high Taxol production. Selected cell lines were then analysed for stability to obtain lines that were stable and produced high volumes of Taxol.

Materials and methods

Establishment and culture of callus lines

Young stems of *Taxus cuspidata* cultivated in the garden of the College of Biological and Agricultural Engineering, Jilin University were collected in June, then washed with soap and water and rinsed with tap water for 3–4 h. The stems were stripped of needles, immersed in 75% ethanol for 30 s, sterilised with 0.1% $HgCl_2$ for 5–7 min, and rinsed 4–6 times with sterile distilled water. Stems were then cut into sections about 1 cm long and inserted into solid B5 medium supplemented with 20 g L^{-1} sucrose, 2.0 mg L^{-1} 1-naphthlacetic acid (NAA) and 100 mg L^{-1} ascorbic acid (pH adjusted to 5.8 with 0.1 mol L^{-1} NaOH). The cultures were placed in a dark incubator at 25 ± 1 °C, and 70 ± 5% humidity. Any explants with bacterial contamination were removed immediately. After 3–5 weeks, callus tissues began to form. Callus tissues were then subcultured on fresh medium every 25 days. FW of the calluses was measured every generation, and a proliferation factor ((final FW − initial FW)/initial FW) of callus FW was calculated.

Cell suspension culture from callus

Selected callus tissues of 5 g were added to 250 mL Erlenmeyer flasks containing 50 mL liquid B5 medium supplemented with 20 g L^{-1} sucrose, 2.0 mg L^{-1} NAA, and 100 mg L^{-1} ascorbic acid. The cultures were maintained in a rotary shaker at 120 rpm and 25 ± 1 °C in darkness. The suspension cells were subcultured every 2 weeks. The cultures were then allowed to stand so that the supernate could be removed and replaced with the same amount of fresh medium.

Establishment of cell lines from single cell

Suspension cells that had been subcultured three times were filtered through a 0.175 × 0.175 mm stainless steel mesh, then through a 0.075 × 0.075 mm stainless steel mesh. Most of the obtained cells were single cells; a few cell clusters consisted of 2–5 cells. Next, the cell density was determined using a hemacytometer. Then, the suspension cells were diluted to appropriate concentrations for standard plate culture, conditioned culture, and nursing culture. The inducing medium was used for standard plate cultures; autoclaved medium was cooled to about 35 °C and fully mixed in a 1:1 volume with a single cell suspension, which was then quickly spread on a Petri dish. The lidded dish was then sealed with parafilm. Cultures were maintained in a growth chamber at 25 °C in darkness. Following 15 min centrifugation ($2000\times g$) at 20 °C, the supernate of the single cell suspension was fully mixed in a 1:1 volume with inducing medium, which was cooled to about 35 °C, and used as a conditioning medium. Afterward, the conditioning medium was mixed in a 1:1 volume with a single cell suspension at an appropriate concentration. Finally, the cells were cultured in a Petri dish in a growth chamber at 25 ± 1 °C, in darkness.

As to the nursing culture, the single cell suspension was fully mixed at a 1:1 ratio with inducing medium that had cooled to about 35 °C, and then the mixed medium was quickly spread into the Petri dish and spread out. After the medium solidified, a round of sterile filter paper was laid on the surface, and a single-cell suspension at an appropriate concentration was placed on the filter paper, covered, and sealed with parafilm. Cultures were maintained in a growth chamber at 25 °C in darkness. We used plating efficiency (PE) to measure the effect of the initial number of cells in the culture on the number of cell clusters formed during plate culture (number of cell clusters formed/number of initial cells) × 100%).

Selection and subculture of cell lines

After 5–6 weeks of single-cell culturing, cell lines of various colours, shapes, and growth states were subcultured on the inducing medium supplemented with 0.5 g L^{-1} casamino acids. Cultures were maintained in a growth chamber at 25 ± 1 °C, in darkness. There were three subcultures over 3 months. Before each transfer, cell lines with good growth status were selected for further culturing; any cell lines with slow growth, browning, or contamination were discarded. Each selected cell line was considered as the first generation, divided into two halves, half for

subculturing, and the other half for determining growth rate and Taxol content. Next, cell lines with high Taxol content and good growth after repeated selections were cultured over an extended time. Finally, in a stability analysis, the high-yield cell lines were subcultured in the solid and liquid subculture media, and the rate of increase in fresh mass (FW) and Taxol content were determined.

Determination of growth rate

FW of suspension cells was determined using 1 mL of culture in a preweighed Eppendorf tube. The culture in the tubes was centrifuged at $2000\times g$ for 15 min, then the supernate was removed with a pipette, and the culture plus Eppendorf tube reweighed and the mass of the tube was subtracted from the combined mass to give FW of the cells.

Extraction and determination of Taxol

The callus tissues were dried at 40 °C, and then fully ground with a mortar and pestle. The dried, powdered callus tissues, weighing 100 mg, in 5 ml ethanol–dichloromethane (1:1) were ultrasonicated for 1 h at 200 W, then centrifuged at $2000\times g$ for 15 min (25 °C). Next, the supernate was collected and dried using a rotary evaporator at 25 ± 1 °C. The residue was dissolved in 5 ml ethanol for assay. In addition, Taxol was extracted from the cell suspensions as follows: 10 ml suspension cell culture was centrifuged at 2000×g for 15 min (25 °C). The supernate was collected, and the pH was adjusted to 7.0. The supernate was then thrice extracted with 3 mL ethyl acetate. The ethyl acetate phase was collected and evaporated *in vacuo* and dissolved in 5 ml methanol for determination. Meanwhile, the pelleted cells obtained after centrifugation were also extracted for Taxol as done for callus tissues. Taxol content was determined as described by Wang et al. (2016).

Stability analysis of selected cell lines

Generally, most of the selected cell lines were not stable, so high-yielding cell lines were analysed for stability by growing them on solid and liquid B5 media with subculturing every 2 weeks for 44 weeks. Percentage growth in FW was calculated as [(FW of each generation − initial FW)/initial FW] × 100%.

Statistical analysis

Data are the average of 3–5 determinations ± standard deviation (SD). The dispersion of data in each group was represented by SD.

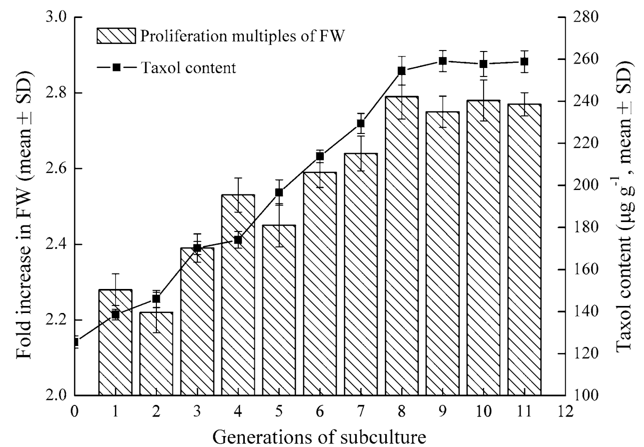

Fig. 1 Fresh mass increase (FW) and Taxol content of callus generations. Data are the average of five determinations; *bars* represents ± SD

Results and discussion

The effect of subculture on growth and Taxol content of callus

As shown in Fig. 1, when the subculture time was less than eight generations, callus FW increased from 2.28- to 2.79-fold with successive generations of subculture. In the subculture process, the growth rate and colour of callus were unstable, perhaps due to an effect of the environment and culture medium on the initial callus. After the eighth generation, the growth rate of the callus was relatively stable. The average proliferation in FW of generations9, 10 and 11 was 2.75-, 2.78-, and 2.77-fold, respectively. Continued subculture did not significantly increase the FW of callus beyond the ninth generation. With each generation of subculturing, Taxol production increased gradually, reaching the highest yield of 259.14 μg g^{-1} DW (dry mass) during the ninth generation of callus. As for fresh mass, Taxol production did not continue to increase beyond the level of the ninth generation; Taxol levels for generations 10 and 11 were on average 258.78 μg g^{-1} DW and 258.90 μg g^{-1} DW, respectively. When the growth rate and Taxol production of *Taxus cuspidata* callus were taken into account, the callus of the ninth generation could be used to select stable, high Taxol-producing cell lines.

The effect of different culture methods on single-cell cloning

The plating efficiencies (PEs) after 40 days of single-cell cloning in normal plate culture, conditioned culture, and nursing culture are shown in Table 1. With a decrease in the initial cell density used, the PE for the three methods

Table 1 Effects of different culture methods and initial cell density (0.5–5.0 × 10^3 mL^{-1}) on plating efficiency (PE)

Culture method	Mean plating efficiency ± SD (%)					
	0.5 × 10^3 mL^{-1}	1 × 10^3 mL^{-1}	2 × 10^3 mL^{-1}	3 × 10^3 mL^{-1}	4 × 10^3 mL^{-1}	5 × 10^3 mL^{-1}
Standard plate	0	0.48 ± 0.01	1.68 ± 0.04	3.54 ± 0.08	8.28 ± 0.16	14.04 ± 0.28
Conditioned	2.00 ± 0.05	4.30 ± 0.09	6.30 ± 0.14	9.53 ± 0.21	14.35 ± 0.29	16.12 ± 0.32
Nursing	3.20 ± 0.07	4.00 ± 0.08	5.45 ± 0.13	9.83 ± 0.19	13.88 ± 0.26	16.92 ± 0.34

Data are the average of five determinations ± SD

Table 2 Characteristics of selected cell lines after three rounds of subculturing

Cell line	Growth rate of FW (%)	Taxol content (% DW)	Colour and morphological features
1	129.5	0.0282	Light brown, dense, and massive
2	194.6	0.0277	Light brown, friable, and granular
3	298.8	0.0337	Light yellow, friable, and granular
4	289.4	0.0313	Light brown, dense, and massive
5	346.3	0.0448	Light yellow, friable, and granular
6	369.6	0.0424	Light yellow, friable, and granular
7	239.2	0.0227	Light brown, dense, and massive
8	266.7	0.0315	Light yellow, dense, and massive
9	184.3	0.0280	Light brown, friable, and granular
10	332.4	0.0424	Light yellow, friable, and granular
11	364.8	0.0441	Light yellow, friable, and granular
12	382.5	0.0477	Light yellow, friable, and massive
13	242.3	0.0298	Light brown, dense, and massive
14	349.2	0.0415	Light yellow, friable, and granular
15	296.1	0.0351	Light yellow, friable, and massive
16	222.7	0.0286	Light yellow, dense, and massive
17	273.3	0.0335	Light yellow, dense, and massive
18	383.7	0.0452	Light yellow, friable, and granular
19	331.9	0.0417	Light yellow, friable, and granular
20	403.4	0.0460	Light yellow, friable, and massive
21	409.2	0.0428	Light yellow, friable, and granular
22	394.5	0.0431	Light yellow, friable, and granular
23	338.4	0.0369	Light yellow, friable, and massive
24	362.6	0.0467	Light yellow, friable, and granular
25	272.3	0.0304	Light brown, friable, and granular

gradually decreased. The effects of conditioned culture and nursing culture were similar, but the gap between the normal plate culture and the two methods was significant, and increased with decreasing cell density. When the cell density was reduced to 1 × 10^3 cells mL^{-1}, the PE of conditioned culture and nursing culture was eight times that of the normal plate culture. There were no cell clones in normal plate culture, but there were still a few cell clones in the conditioned culture and nursing culture when the initial cell density was decreased to 0.5 × 10^3 cells mL^{-1}.

In maintaining growth and inducing cell division, endogenous metabolic substances need to reach a certain threshold, or cell division will be arrested. The PE at high cell densities was relatively high, which may be because this threshold is easily reached. For the same cell density, the PE of conditioned culture and nursing culture is higher than in normal plate culture, perhaps due to the large amounts of metabolites (such as conditioning factor) in the two media promoting cell growth and division.

As Table 1 shows, the PE increased with increasing cell density; however, when the cell density was too high, the distance between cell clusters was very small, which increased the difficulty of isolating single-cell clones. Therefore, according to the appropriate PE and the growth state of cells, the initial cell density for inoculation was set to 3 × 10^3 cells mL^{-1}. Moreover, the use of filter paper in

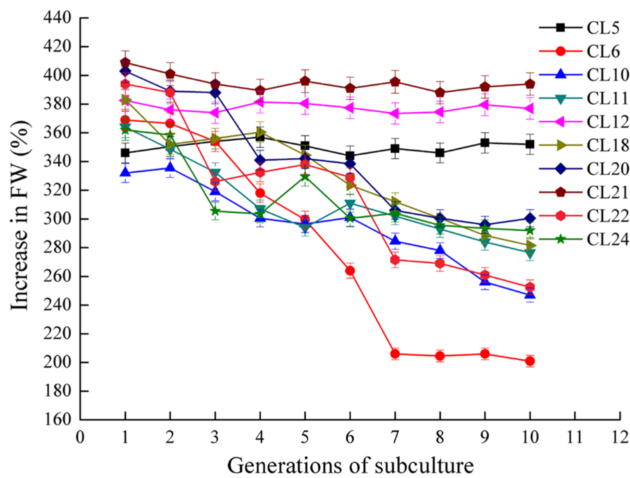

Fig. 2 Changes in fresh mass (FW) of selected cell lines for each generation on B5 solid medium

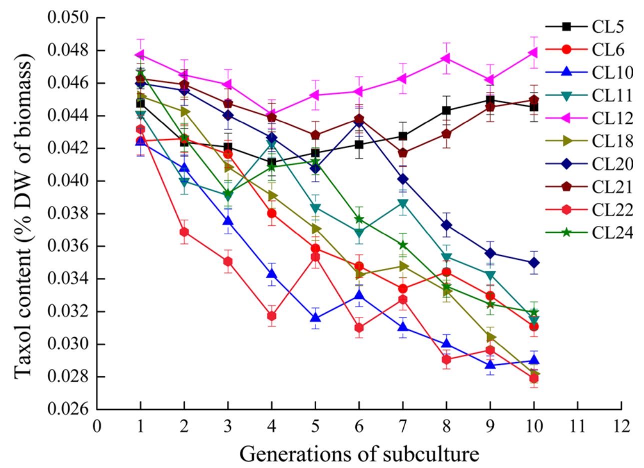

Fig. 4 Taxol content of selected cell lines for each generation on B5 solid medium

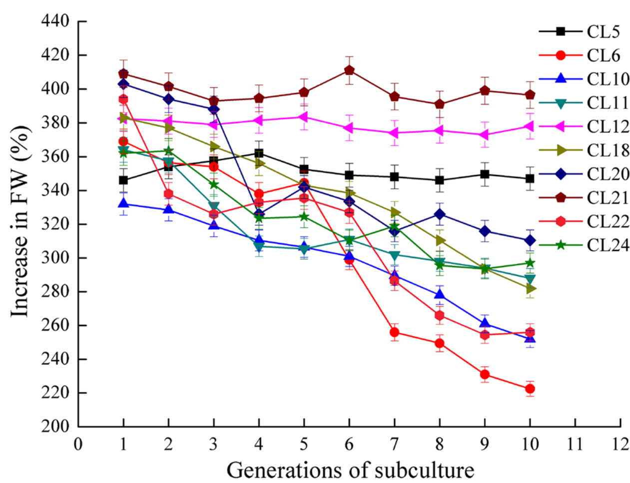

Fig. 3 Changes in fresh mass (FW) of selected cell lines for each generation in the B5 liquid medium

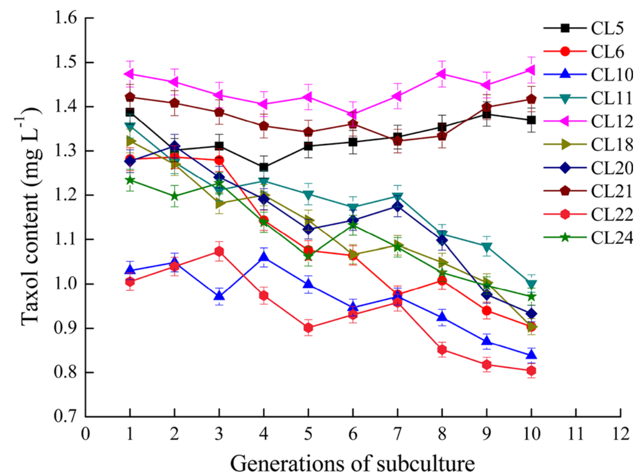

Fig. 5 Taxol content of selected cell lines for each generation in B5 liquid medium

the nursing culture increased the chance of contamination. So conditioned culturing was used for single-cell cloning.

Growth state analysis of selected cell lines

Through the use of conditioned culture, followed by continuous subculture, 97 cell lines were obtained. On the basis of colour and morphological characteristics, 25 cell lines were preliminarily selected. Growth rate and Taxol content were also determined. As shown in Table 2, most of the 25 cell lines were light yellow, friable, and granular. Apparently, the deeper the colour of a cell line, the slower its growth, and Taxol levels were lower. Based on lower growth in FW and Taxol production, 10 cell lines (CL5, CL6, CL10, CL11, CL12, CL18, CL20, CL21, CL22, and CL24) were culled.

Stability of selected cell lines

Figure 2 shows that the FW increases in CL5, CL12, and CL21 on solid B5 medium were relatively stable and grew faster than the other lines. CL6, CL10, CL11, CL18, CL20, CL22, and CL24 had notable decreases or fluctuations in FW, indicating that they were unstable.

As shown in Fig. 3, the FW increases of CL5, CL12, and CL21 in liquid medium were relatively stable, and FW was greater than that for other cell lines. Although the FW increases for CL6, CL11, CL20, CL22, and CL24 began to stabilise after FW decreased, the overall trend was not stable at lower increases in FW. It was clear that FW of CL10 and CL18 not only increased slowly but was still tending to decrease after 10 generations.

Figures 4 and 5 show the change in Taxol production for cell lines cultured on solid and in liquid B5 media,

respectively. Taxol production by CL5, CL12, and CL21 was higher than for other cell lines. Production by these three lines was also relatively stable, whereas production by the other lines decreased with subculturing.

In the stability analysis, the CL5, CL12, and CL21 cell lines were high yielding and stable in both biomass growth and Taxol production. Following the subculture of selected cells, growth and Taxol production were not stable. Several reasons could explain this instability. Plant cells in vitro are easily mutated, and the mutation could lead to some of the cells being stable. Because the cell used for single-cell cloning could comprise 2–5 cells, the difference in characteristics could intensify with subcultivation, causing whole cell lines to become unstable. In addition, browning, a common factor in plant tissue culture, occurred in some cell lines, some cells grew slowly or died. The change in Taxol content is unavoidable, but we can select stable and high Taxol-producing cell lines to subculture. Cryopreservation should also help to overcome this difficulty.

Conclusions

As determined through 11 subcultures of *Taxus cuspidata* callus, the growth in FW and level of Taxol production were stable by the ninth generation. FW increased 2.77-fold, and Taxol production was 259.14 $\mu g\ g^{-1}$ DW. So the callus from the ninth generation could be used for selecting high Taxol-producing cell lines. After comparing normal plate culture, conditioned culture, and nursing culture, conditioned culture was used for single-cell cloning and an initial cell density 3×10^3 cells mL^{-1} was appropriate. After a stability analysis of 10 high-yielding cell lines, lines CL5, CL12, and CL21 were selected; Taxol contents were 0.0448, 0.0477, and 0.0428% DW, respectively. The proliferation increase of CL5, CL12, and CL21 was 346.3, 382.5, and 409.2%, respectively. These three stable, high yielding cell lines are therefore candidate sources for the mass producing Taxol and promoting commercial production using cell culture. For obtaining a more stable, higher yield from such cells, selection and cultures methods will continue to be investigated in future research. This study on selection methods for high Taxol-producing cell lines of *Taxus cuspidata* lays a foundation for selection of other plant cells. Further research, such the optimising the medium and culturing conditions, is now needed to move beyond the laboratory stage into full-scale production.

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Author's contributions S W. and Y. Z designed the experiments. H. W., T. L. and C. L. performed experiments. H. W. analysed the results and wrote the manuscript.

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