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Effect of Subculture and Elicitation on Instability of Taxol Production in *Taxus* sp. Suspension Cultures

Beum Jun Kim, Donna M. Gibson,† and Michael L. Shuler*

School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, New York 14853, and USDA, ARS, U.S. Plant, Soil, and Nutrition Lab., Ithaca, New York 14853

The production of secondary metabolites through plant cell suspension cultures is challenging because the level and pattern of production is often unstable and unpredictable. To investigate the factors affecting instability of secondary metabolite production, high Taxol (paclitaxel)-producing *Taxus* cultures induced by methyl jasmonate elicitation and their low Taxol-producing counterparts were compared with respect to growth and Taxol production kinetics. With *Taxus* subcultures we observe alternating states of high and low productivity. Parental cultures and their subcultures from five different cell lines were used to test whether a high-producing culture grows more slowly or dies more rapidly than a low-producing one. These cell lines were of three types: (1) Taxol-producing with and without methyl jasmonate, (2) Taxol-producing only upon elicitation, and (3) nonproducing. High-producing cultures show growth inhibition upon subculture, whereas nonproducing elicited cultures show little growth inhibition. Thus, growth inhibition is primarily due to Taxol or taxane accumulation and not a direct result of methyl jasmonate treatment. Through media exchange between high- and low-producing cultures, it appears that culture components generated by cells alter culture properties. To assess variability as a function of culture lineage, two groups of replicate cultures were generated either with a mixing of the parental flasks or segregation of parental flasks at each subculture. Although parental culture mixing did not reduce flask-to-flask variation, the production level of Taxol in subcultures resulting from mixing inocula was sustained at a higher level relative to segregated subcultures. The results are consistent with the possibility of cell signaling within the population that can induce Taxol production.

Introduction

Many secondary metabolites derived from plants have been used as pharmaceuticals. Among these plant natural products, Taxol (paclitaxel) has been a highly successful anticancer drug since it was initially approved for the treatment of breast and ovarian cancers (*1*). Other molecular targets for taxanes, such as multidrug resistance inhibition, apoptosis inhibitor binding, and treatments for non-small-cell lung cancer and AIDS-related Kaposi's sarcoma, are still being investigated (*2*−*4*). Thus, demands on the supply of Taxol continue as a result of its expanded use in earlier intervention therapies and its use in combination with other chemotherapeutic agents.

The supply of Taxol was initially limited because only a small amount of the substance was obtained from tree bark (*5*), and the complexity of Taxol's structure has hampered efforts to find a method to achieve an economically feasible route via total synthesis. Viable commercial production was accomplished through a semisynthesis method, through which more abundant natural non-side chain taxanes were converted to Taxol (*6*). However this methodology requires the continued use of precursors from natural sources and significant amounts of solvents, both of which present environmental problems. Another alternative is plant cell suspension culture, which has several advantages: this method is similar to well-established procedures that have been used successfully in bacterial fermentation; it facilitates basic studies on Taxol biosynthesis (*7*); it appears to accumulate compounds not found in natural abundance in bark and needles of plants (*8*); and this process can provide an environmentally friendly path to a high purity product. Many researchers have tried to develop methods to produce Taxol and to enhance its productivity through a cell suspension system (*9*−*11*). Some of the results have been promising, and commercial level production of Taxol from plant cell culture is now a reality (*12*).

A common problem in the use of plant cell cultures is maintenance of consistently productive cultures; *Taxus* sp. cultures making Taxol often display significant instability in product accumulation (*13*). Gradual loss of production of a secondary metabolite is a common obstacle in developing a large-scale production system (*14*, *15*). An inconsistent production pattern during a subculture complicates the development of an economical process and interferes with basic studies on biosynthesis (*13*, *16*, *17*), especially since there is high variation from flask to flask (*16*). Plant cell suspension cultures are known to be highly sensitive to environmental factors such as initial cell density, temperature, and medium components (*18*).

* To whom correspondence should be addressed. Tel: (607) 255-7577. Fax: (607) 255-9166. Email: mls@cheme.cornell.edu.
† USDA.

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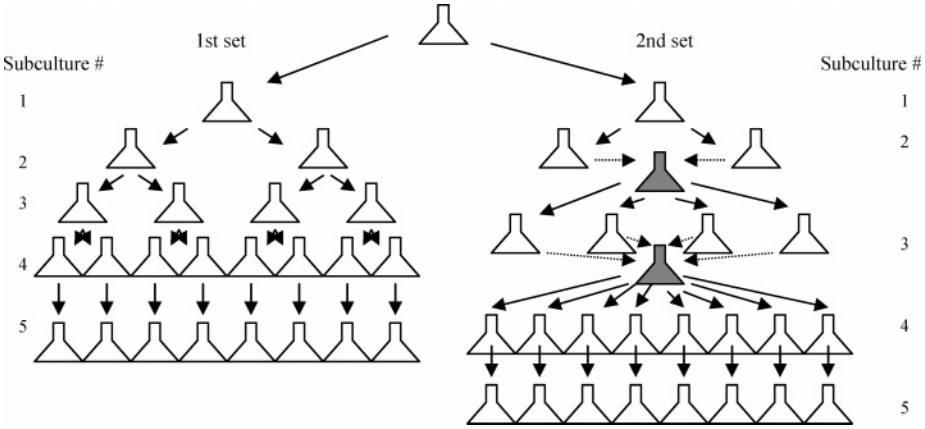

Figure 1. Experimental scheme of obtaining replicate cultures. The gray flasks represent mixing of the parental cultures prior to subculture.

The causes for production instability have been thought to be environmental, genetic, or epigenetic in nature, leading to inhomogeneity within cultures represented by distinct subpopulations (*19*). Others have reported that a secondary metabolite was produced by only a small fraction of cells in the culture and that production enhancement often meant an increase in the proportion of productive cells (*20*). Another possibility is changes in inter- and intracellular communication, since the shift pattern between increases and decreases in the production levels are not simply monotonic (*21*). Rather, it appears to be a coordinated event that is not independent of other cells, which suggests that cells may generate chemical signals for secondary metabolite production within the culture cycle.

On the basis of these observations, we hypothesized that high-producing cells grow more slowly or die more rapidly than their low-producing counterparts. We also considered as a second hypothesis that there is a chemical signal(s) generated by cells requiring a threshold limit to induce secondary metabolite production. In this work, high- and low-producing parental cultures and their subculture lineages were compared to define what factors may contribute to instability and how to minimize instability of production.

Materials and Methods

Cell Maintenance. Cell lines of *Taxus canadensis* CO93P, CO93P2, CO93D, C93AD and *Taxus cuspidata* P93AF were originally developed by R. Ketchum, USDA, ARS, Ithaca, NY (*22*−*24*). All cell lines were subcultured every 2 weeks. Subculture was accomplished by transferring 3.0 mL packed-cell-volume of 14-day-old cells and 7.0 mL of 14-day-old conditioned medium to 40.0 mL of autoclaved fresh B5NB medium in a 125-mL flask with a Bellco (Vineland, NJ) foam cap (*25*). The culture was supplemented with an antioxidant mixture that was filter-sterilized prior to addition at a final concentration of 2.0 mM L-glutamine, 0.285 mM L-ascorbic acid, and 0.26 mM citric acid. All the cultures were incubated at 24 °C in the dark and shaken at 120 rpm.

Culture Comparison. High-producing cultures were initiated by treatment with a final concentration of 100 μM methyl jasmonate as an elicitor at day 7. Progeny cultures were produced by a subculture of parental cultures at day 14. For parental cultures, data were obtained until day 35 at 7-day intervals, following subculture. For progeny cultures derived from a subculture of the parental culture at day 14, data were obtained until day 21 at 7-day intervals.

Media Exchange. Eight replicate cultures were generated after mixing of two parental cultures at subculture. At day 7 after replicate culture generation, four cultures were treated with methyl jasmonate to generate high-producing cultures. At the specified exchange day, all of the media (approximately 30 mL from each culture) were removed with a pipet from each culture, and then remaining cells were washed with 20.0 mL of fresh B5NB medium twice. Media from two high-producing cultures were transferred to two low-producing culture cells (high-to-low) and vice versa (low-to-high). Media from the remaining two high-producing cultures were transferred to original high-producing culture cells, which were considered as high-producing controls (high-to-high). Media from the remaining two low-producing cultures were transferred to original low-producing culture cells, which were considered as low-producing controls (low-to-low).

Fresh Cell Density and Dry Cell Density. To measure cell density at each time interval, 1.0 mL of cell suspension was removed from a culture and transferred to a preweighed Eppendorf tube. After centrifugation at 13,000 rpm for 10 min, all of the liquid was removed using a micropipet, and then the tube was weighed to obtain fresh cell density. The sample was then dried at 80 °C for 1 week and then reweighed to obtain dry cell density.

Extracellular Taxane Analysis. To analyze taxane contents, 1.0 mL of cell-free culture medium was removed at specified time intervals and then freeze-dried using a Savant Speed-Vac (Savant Instruments, Farmingdale, NY) until the pressure dropped below 100 μTorr. The residual pellet was resuspended with 100 μL of methanol acidified with 0.01% acetic acid, sonicated for 30 min in a water bath, and then centrifuged for 15 min at 13,000 rpm. The supernatant was filtered through a 0.2 μm PVDF filter (Gelman Sciences, Ann Arbor, MI) and analyzed on HPLC (*25*, *26*). Three standard taxanes consisting of baccatin III, cephalomannine, and Taxol (Sigma Chemical Co.) were used to generate standard curves, retention times, and spectral comparisons.

Method of Obtaining Replicate Flasks. Two sets of replicated cultures were designed as described in Figure 1. In the first experimental set, all replicated cultures were produced by segregation: at every subculture, two progeny cultures were obtained from one parental culture. At the fourth subculture, eight replicate flasks were obtained, and one-to-one subculture was performed at the fifth subculture. In the second set, all replicate cultures were obtained with mixing: at every

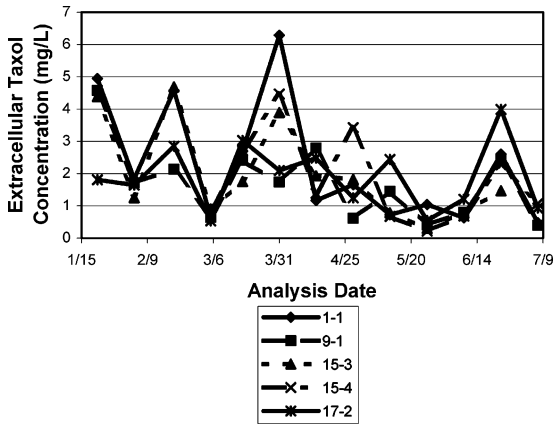

Figure 2. Changes in extracellular production of Taxol over a 6 month subculture cycle with *T. canadensis* CO93P2 cell line. Flask number is in legend.

subculture, all parental cultures were mixed and distributed into progeny cultures. At the fourth subculture, eight replicate flasks were obtained, and also one-to-one subculture was performed at the fifth subculture.

Statistical Analysis. Data were analyzed by both the Wilcoxon rank sum test and one-way ANOVA to test equal mean values ($\alpha = 0.05$) (*27*). Data were also analyzed by the squared ranks test for equal variances ($\alpha = 0.05$) (*28*).

Results and Discussion

Maintenance and Instability Challenge. All cell lines were maintained with biweekly subculture with the production of Taxol checked on day 12 following subculture. The unelicited production of Taxol fluctuated between a high-producing and low-producing state in the majority of individual flask lineages for *T. canadensis* CO93P2 (Figure 2). For example, with flask number 1-1, the production upon subculture changed from 4.95 to 1.83 and then to 4.58 mg/L, etc. In general, the production level with each culture lineage gradually decreased over time (Figure 2), as seen in other plant culture systems (*14*, *15*). Our results show that the subculture variance is high; for example, at 3/31, the highest value is 6.29 mg/L of Taxol, but the lowest value is 1.74 mg/L, resulting in an average of 3.70 mg/L and a variance of 3.44 $(mg/L)^2$. We have observed similar patterns of alternating states and subculture variance with other *Taxus* sp. lines.

Subculture Comparison using Effects of Elicitation. We have delineated three types of cell lines based on the response to methyl jasmonate. The first type can produce Taxol both with and without elicitation, the second type can produce Taxol only with elicitation, and the third type cannot produce Taxol even when treated with elicitors. In the following experiments, we compared growth and Taxol production between elicited and nonelicited cultures of all three types over two generations, using elicitation on day 7 of the original parental line (Table 1).

Taxus canadensis CO93P and CO93P2, characteristic of type 1 cell lines, produce Taxol both with and without methyl jasmonate elicitation. The growth was inhibited in elicited cultures in both the parental culture and the subsequent subculture (Figures 3 and 4). The growth inhibition of elicited *T. canadensis* CO93P2 was evident at day 21 (14 days after elicitation) when high production of Taxol (240.3 ± 30.3 mg/kg cell fresh weight; 5 times higher than control) was first recorded, and continued

Table 1. Experimental Scheme for Comparison between Cultures Elicited with 100 μM Methyl Jasmonate (MJ) and Nonelicited Cultures

type	cell line	parental cultures	progeny cultures	replicate	figure no.
I	*T. canadensis* CO93P2	day 0−35	day 14−35	3	3
	T. canadensis CO93P	day 0−35	day 14−35	3	4
II	*T. cuspidata* P93AF	day 0−35	day 14−35	3	5
III	*T. canadensis* CO93D	day 0−35	day 14−35	3	6
	T. canadensis C93AD	day 0−35	day 14−35	3	7

until end of the culture, at day 35 when the final fresh cell density of the elicited parental culture reached 0.093 ± 0.010 g/mL, whereas that of the nonelicited parental culture was 0.165 ± 0.042 g/mL (Figure 3). The elicited progeny of *T. canadensis* CO93P2 exhibited growth inhibition at the beginning of the subculture, and culture death followed quickly. The final fresh cell density of the nonelicited progeny of *T. canadensis* CO93P2 was 0.141 ± 0.002 g/mL, whereas that of the elicited progeny culture was 0.055 ± 0.008 g/mL, which was one-third of its counterpart.

The growth patterns of the parents and progenies in both elicited and nonelicited cultures of *T. canadensis* CO93P were similar to those of *T. canadensis* CO93P2 with lower final cell densities (Figure 4), as may be expected since are both type 1 cultures. As seen from Figure 4 the final fresh density of the nonelicited parental and progeny cultures is about twice that of the corresponding elicited parental and progeny cultures. While the elicited parental cultures continue to produce Taxol, the elicited subcultures did not produce as much Taxol as either the elicited or the nonelicited parental cultures for the same period of time (21 days). These results show that either methyl jasmonate or high Taxol levels or a combination of both resulted in growth inhibition.

The second type of culture, as illustrated by *T. cuspidata* P93AF, produces Taxol only upon methyl jasmonate elicitation. Both the elicited parental culture and its subculture showed growth inhibition (Figure 5). The final

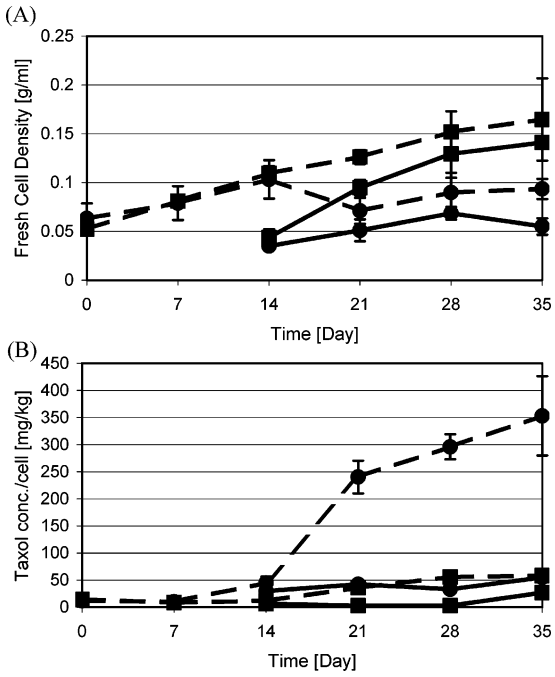

Figure 3. Comparison of cell density (A) and Taxol production (B) with *T. canadensis* CO93P2 cultures elicited with 100 μM methyl jasmonate (MJ) or nonelicited (triplicates for elicited and nonelicited cultures): (■) nonelicited culture, (●) elicited culture, (- - -) parent culture, (—) progeny culture.

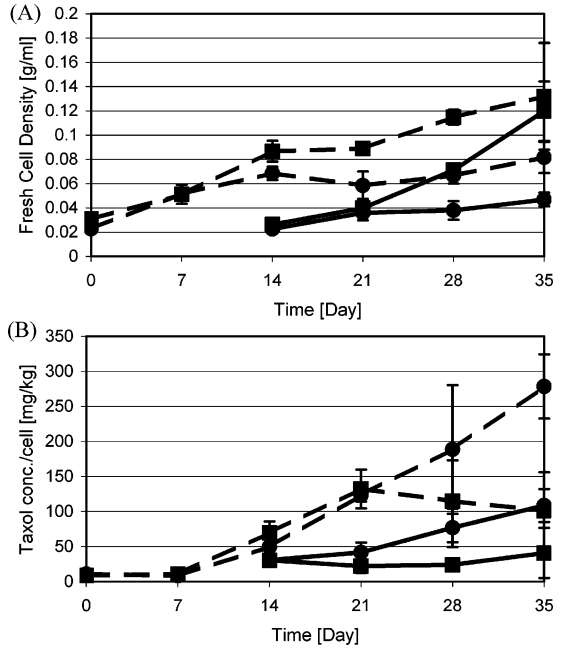

Figure 4. Comparison of cell density (A) and Taxol production (B) with *T. canadensis* CO93P cultures elicited with 100 μM methyl jasmonate (MJ) or nonelicited: (■) nonelicited culture, (●) elicited culture, (- - -) parent culture, (—) progeny culture.

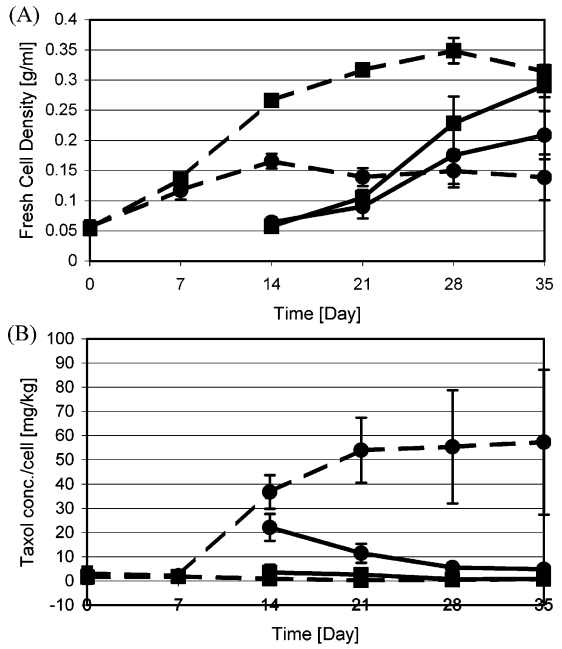

Figure 5. Comparison of cell density (A) and Taxol production (B) with *T. cuspidata* P93AF cultures elicited with 100 μM methyl jasmonate (MJ) or nonelicited: (■) nonelicited culture, (●) elicited culture, (- - -) parent culture, (—) progeny culture.

fresh cell density of the nonelicited parental culture of *T. cuspidata* P93AF reached 0.314 ± 0.011 g/mL, whereas that of the elicited parental culture was only 0.139 ± 0.038 g/mL. The final fresh cell density of the nonelicited progeny culture of *T. cuspidata* P93AF was 0.291 ± 0.019 g/mL, and that of the elicited progeny culture was 0.209±0.040 g/mL. *T. cuspidata* P93AF grew much better than the other cell lines tested, and thus the growth inhibition in the elicited cultures of *T. cuspidata* P93AF is less obvious than those in other cell lines. The elicited subculture did not produce Taxol, and the elicited parental culture barely retained the ability to produce Taxol

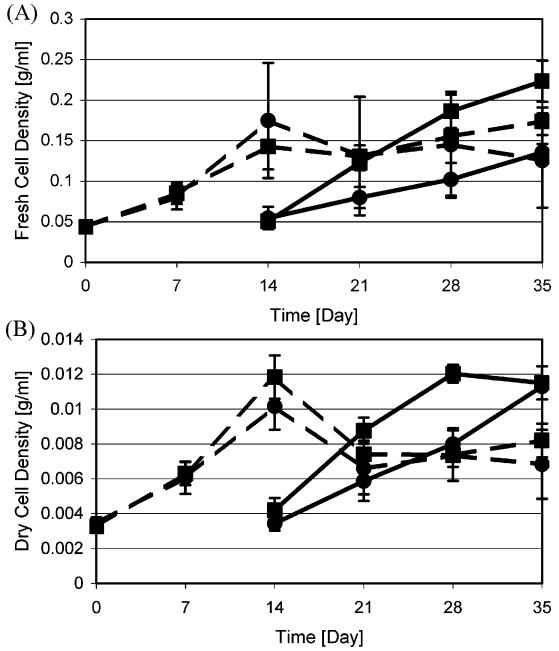

Figure 6. Alterations in culture growth of control and methyl jasmonate elicited cultures of *T. canadensis* CO93D over a subculture cycle ((A) fresh cell density and (B) dry cell density): (■) nonelicited culture, (●) elicited culture, (- - -) parent culture, (—) progeny culture.

after subculture. Although the characteristics of the growth of the elicited culture did not change upon subculture, the pattern of production of the secondary metabolite was altered after subculture.

To determine if methyl jasmonate itself could affect the growth of *Taxus*, the third type of cell lines, which do not produce Taxol either with or without elicitation, were tested using *T. canadensis* CO93D and C93AD derived from formerly producing cultures. Over a time course of 35 days, fresh cell densities of the elicited and nonelicited parental cultures were not affected by methyl jasmonate; both elicited and nonelicited cultures of CO93D show the same growth pattern through day 28 (Figure 6). At day 35, the nonelicited culture sustained biomass levels while the elicited culture displayed a slight loss in cell density. However, the growth of the elicited progeny culture was consistently lower than for the nonelicited subculture, and a small amount of taxanes was produced, indicating that the growth may have been slightly inhibited as a result of taxane production. Although the growth patterns of elicited and nonelicited C93AD parental cultures were similar, the elicited progeny culture exhibited a higher cell concentration than the nonelicited progeny culture, implying methyl jasmonate itself might not be a major reason for growth inhibition in *Taxus* sp. cultures (Figure 7).

From the comparison between high-producing and low-producing cultures in both the parental lineages and their subcultures, we suggest that the growth inhibition is causally related to Taxol production and methyl jasmonate elicitation affects both growth and secondary metabolite production: once the Taxol production mechanism is initiated, the growth is severely depressed for a long period even upon subculture. The observations suggest that once the growth inhibition mechanism occurs, it is not easily reversed, even by transferring of the cells to fresh medium. The growth inhibition may be an indication of cellular differentiation leading to cell death occurring in the secondary metabolite-producing

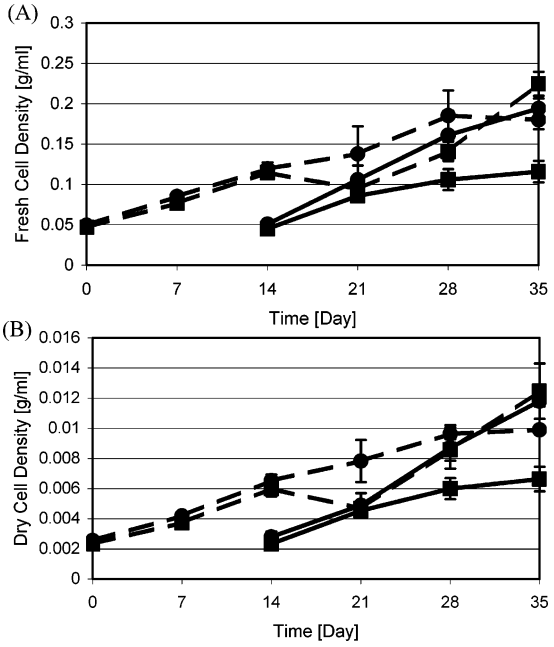

Figure 7. Alterations in culture growth of control and methyl jasmonate elicited cultures of *T. canadensis* C93AD over a subculture cycle ((A) fresh cell density and (B) dry cell density): (■) nonelicited culture, (●) elicited culture, (- - -) parent culture, (—) progeny culture.

cells within the culture. In contrast, the secondary metabolite production can be dependent on the particular culture condition and environmental conditions, i.e., methyl jasmonate elicitation, since not all cell lines are able to produce secondary metabolites while growth was somewhat affected by elicitation. As shown, high-producing parental cultures maintained the ability to produce Taxol, although subcultures of high-producing parents can produce little or no Taxol. One possibility is that there may be a chemical signal generated by cells within the culture that induces a change in culture state leading to the production of Taxol. In the high-producing parental culture, the level of the chemical signal may be sufficient to switch cells into a high-producing state, and hence the high-producing parental culture is able to continue high production of Taxol. At the same time, the chemical signal may be diluted by new medium in the subcultures from high-producing cultures, and these cultures may produce less Taxol but have the same growth characteristic as the parental cultures.

Subculture Comparison using Effects of Media Exchange. To test if a cell-generated compound could alter the cell growth and Taxol production, media exchange was conducted using high-producing *T. cuspidata* P93AF cultures elicited by methyl jasmonate at day 7 and low-producing cultures (Table 2). When the media were exchanged at day 8, both high-to-low and low-to-high cultures appeared to be affected by methyl jasmonate elicitation since the growth patterns of high-to-low, low-to-high, and high-producing (high-to-high control) cultures were similar (Figure 8A). When the media were exchanged at day 11, the results were quite different (Figure 8B). The growth of high-to-low cultures recovered, unlike the cultures exchanged at day 8, implying that the effect by volatile methyl jasmonate became minimal and cell-generated medium components might be important. Their growth rates were intermediate between the growth of high-producing (high-to-high control) cultures and that of low-producing (low-to-low control) cultures and became statistically equivalent. During these media

Table 2. Experimental Scheme for Media Exchange

cell line	media exchange day	replicate	figure no.
T. cuspidata P93AF	8	2	8A
	11	2	8B

exchange experiments, some cultures produced low levels of taxanes but not Taxol, which complicates interpretation of response in these cultures. When the experiments were done, the cell line was not in a producing state. However, cell-generated compounds that were produced between day 8 and day 11 affect the growth of *T. cuspidata* P93AF.

Conventional clonal selection has been carried out on the basis of the productivity of the culture because, with unselected cultures, the production declines and eventually stops (*14*). In *Taxus* cultures, this selection method may not be an effective means to maintain productivity because cultures selected solely as high-producing cultures may die more rapidly. Cell growth and factors that may trigger cell growth and differentiation should be considered along with productivity as selection criteria for long-term stable production of taxanes.

Flask Variation. Because plant cell suspension culture lines appeared to show a large variation in Taxol production from one flask to another, we designed a subculture scheme to assess inherent variation during subculture by testing the hypothesis that replicate flasks from the same parental culture would be expected to share a similar pattern in cell growth and secondary metabolite production. To obtain replicate cultures, two sets of cultures were produced over a 12-week period as described (Figure 1). All cell density data and Taxol production data were collected at day 14 following subculture. Eight culture flasks from each group using *T. cuspidata* P93AF were compared at the fourth subculture. There were no statistical differences between the two sets of replicate flasks; the average fresh cell density of segregated cultures was 0.197 ± 0.031 g/mL, and that

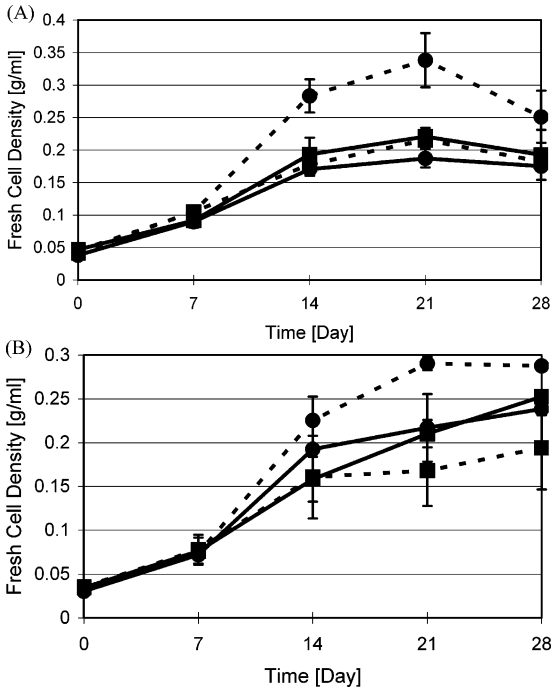

Figure 8. Changes in fresh cell densities after media exchange at day 8 (A) and day 11 (B) with *T. cuspidata* P93AF: (—■—) high-to-low, (···■···) high-to-high, (—●—) low-to-high, (···●···) low-to-low.

Table 3. Comparisons of Fresh Cell Densities, Taxol Contents, and Their Variance at the Fourth and Fifth Subculture Cycles between Subcultures Produced by Segregation or Mixing with *T. cuspidata* P93AF or *T. canadensis* C93AD[a]

T. cuspidata P93AF		fresh cell density [g/mL]		variance [(g/mL)^2]	
subculture	no. of flasks	segregation	mixing	segregation	mixing
fourth	8	0.197 ± 0.031^a	0.211 ± 0.018^a	0.00095^a	0.00031^a
fifth	8	0.176 ± 0.013^a	0.189 ± 0.016^a	0.00016^a	0.00027^a
T. cuspidata P93AF		extracellular Taxol [mg/L]		variance [(ml/L)^2]	
subculture	no. of flasks	segregation	mixing	segregation	mixing
second	2	4.18 ± 5.91^a	6.59 ± 4.16^a	$34.9^{n/a}$	$17.3^{n/a}$
third	4	6.60 ± 4.33^a	11.76 ± 3.68^b	18.7^a	13.5^a
fifth	8	1.94 ± 1.73^a	1.40 ± 1.27^a	2.99^a	1.61^b
T. canadensis C93AD		fresh cell density [g/mL]		variance [(g/mL)^2]	
subculture	no. of flasks	segregation	mixing	segregation	mixing
fourth	8	0.225 ± 0.022^a	0.230 ± 0.016^a	0.00048^a	0.00027^a
fifth	8	0.186 ± 0.019^a	0.194 ± 0.018^a	0.00038^a	0.00032^a
T. canadensis C93AD		fresh cell density [g/mL]		variance [(g/mL)^2]	
subculture	no. of flasks	segregation	mixing	segregation	mixing
fourth	8	0.172 ± 0.023^a	0.168 ± 0.016^a	0.00051^a	0.00025^a
fifth	8	0.160 ± 0.025^a	0.169 ± 0.018^a	0.00060^a	0.00033^a

[a] Data with the same letter are not statistically different at $\alpha = 0.05$.

of mixed cultures was 0.211 ± 0.018 g/mL (Table 3). The variances were analyzed statistically. The variance of cell density for segregated cultures was 0.00095 (g/mL)^2, whereas that of mixed cultures was 0.00031 (g/mL)^2, showing no statistical difference. Initially we designed the experiment to the fifth subculture with a simple one-to-one subculture to assess how long the mixing effect would persist. The average fresh cell density of segregated cultures was 0.176 ± 0.013 g/mL, whereas that of mixed cultures was 0.189 ± 0.016 g/mL, which were statistically equivalent (Table 3). The variance of segregated cultures was 0.00016 (g/mL)^2, while that of mixed cultures was 0.00027 (g/mL)^2, showing no statistical difference. Because the cell line *T. cuspidata* P93AF produces Taxol only with elicitation, extra culture sets were used to assess Taxol production by generation at the second, third, and fifth subcultures. The average Taxol amount in segregated cultures at the third subculture was 6.60 ± 4.33 mg/L, and that in mixed cultures at the third subculture was statistically higher at 11.8 ± 3.68 mg/L ($p = 0.039$). By the fifth subculture where we used a one-to-one subculture, Taxol production had decreased significantly in all flasks. The average Taxol concentration in segregated cultures was 1.94 ± 1.73 and 1.40 ± 1.27 mg/L in the mixed cultures (Table 3); these values were not statistically different. Clearly the variance among flasks is much greater with respect to Taxol production than for growth.

Taxus canadensis C93AD was also used to test equal variance between the two methods of subculturing. In duplicate experiments, test statistics (999 at the fourth subculture in the first, 916 at the fourth subculture in the second, 770 at the fifth subculture in the first, and 1000 at the fifth subculture in the second) in both cases were not within the rejection region (lower than 570 or higher than 1071), indicating statistical equivalence at $\alpha = 0.05$ (Table 3).

Mixing of inocula for subcultures did not reduce the variance among the replicated flasks based on cell growth, suggesting that the variation is inherently induced by subculture, regardless of the method used. Variance in growth, however, is small, although Taxol production was improved with mixing for earlier subcultures. A possible explanation is that chemical signals are involved in this process: mixed cultures may achieve greater overall concentrations of signal compounds in every flask as a result of the combination of inocula than segregated cultures do and thus have a more uniform concentration of signal compounds in every flask. Thus mixing may improve production if the response to the signal compound is nonlinear and saturable.

Cell viability and apoptosis may be keys to understanding unstable production of secondary metabolites. Production of defensive compounds and pathways such as for taxanes are related closely to the cell death mechanism in plants. It was reported that certain fatty acids in octadecanoid pathways for signaling defense, such as those generating jasmonic acid, could induce apoptosis (*29*), and apoptosis can be a mechanism that is activated along with plant defense mechanisms. Ma et al. (*30*) reported that apoptosis was a major death mechanism in *T. chinensis* suspension culture. The same group also reported that a crude extract from *Fusarium oxysprum* induced a significant amount of cell apoptosis in *T. chinensis* suspension culture, which was correlated with Taxol production (*31*). Yuan and colleagues suggest that reactive oxygen species generated during elicitation cause both Taxol production and apoptosis (*32*−*34*). These reactive oxygen species are too short-lived to be the chemical signal we suggest, but other intermediates in the elicitation pathway may be involved. If methyl jasmonate or other signal compounds that elicit Taxol production induce apoptosis, it may make the production of Taxol with a cell suspension culture through conventional selection an inherently unstable process.

Using various viability tests and apoptosis analysis by DNA laddering pattern, evaluation of death kinetics of high-producing *Taxus* cultures was reported in our laboratory for high- and low-producing cultures using *T. cuspidata* P991, which is a type 1 cell line recovered from a nonproducing state (*35*). A double staining method using fluorescein diacetate and phenosafranin resulted in the observation of a viability reduction in high producing cultures initiated with methyl jasmonate treatment, as well as growth reduction and high Taxol production. As a control, *T. canadensis* CO93D was tested, resulting in the observation that viabilities of elicited and nonelicited cultures were similar. DNA laddering was only found

in late culture period (28−42 days) in both methyl jasmonate elicited *T. cuspidata* P991 and *T. canadensis* C93AD under conditions in which it was a Taxol-producing line. In conclusion, growth and viability reduction in high-producing cultures appear to be related to high Taxol production. However, apoptosis is not likely to be a primary death mechanism in these cultures.

Our data indicate, however, that although growth was causally related to Taxol production, it is not the only factor involved in triggering secondary metabolite production. The media exchange and flask variation experiments indicate that factors other than methyl jasmonate appear to have effects on Taxol production, and hence there may be other signaling components that may affect population shifts, leading to secondary metabolite accumulation.

Several protein signal compounds have been isolated in plants and studied analogously to animal protein hormones (*36*, *37*). The isolation and characterization of extracellular signal compounds could be a critical and relatively unexplored approach to understanding secondary metabolism and contribute to production enhancement in plant suspension cultures.

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