Published in final edited form as:

Plant Cell Tissue Organ Cult. 2013 March 1; 112(3): 303–310. doi:10.1007/s11240-012-0237-3.

Cellular aggregation is a key parameter associated with long term variability in paclitaxel accumulation in *Taxus* suspension cultures

Rohan A. Patil, Martin E. Kolewe, and Susan C. Roberts*

Department of Chemical Engineering, Institute for Cellular Engineering, University of Massachusetts, Amherst, MA 01003-9303

Abstract

Plant cell cultures provide a renewable source for synthesis and supply of commercially valuable plant-derived products, particularly for secondary metabolites. However, instability in product yields over multiple passages has hampered the efficient and sustainable use of this technology. Paclitaxel accumulation in *Taxus* cell suspension culture was quantified over multiple passages and correlated to mean aggregate size, extracellular sugar level, ploidy, and cell cycle distribution. Paclitaxel levels varied approximately 6.9-fold over the six-month timeframe investigated. Of all of the parameters examined, only mean aggregate size correlated with paclitaxel accumulation, where a significant negative correlation ($r = -0.75$, $p < 0.01$) was observed. These results demonstrate the relevance of measuring, and potentially controlling, aggregate size during long term culture passages, particularly for plant suspensions where industrially relevant secondary metabolites are not pigmented to enable rapid culture selection.

Keywords

Plant cell culture; bioprocess; cellular aggregation; paclitaxel; *Taxus*; ploidy; DNA content

Introduction

In spite of the demonstrated effectiveness and past successes of plant-based natural products, particularly as chemotherapeutics (Cragg et al. 2009), pharmaceutical companies have had a declining interest in screening for natural products over the past couple of decades (McChesney et al. 2007; Harvey 2008). One of the primary reasons for this decline is the issue of drug product supply, including the economic considerations pertaining to commercialization of a particular supply route. Plant suspension cell culture technology has proven successful for the synthesis of natural products in a controlled environment, with several products supplied commercially at a large scale (Eibl and Eibl 2002; Kolewe et al. 2008). Nevertheless, the use of plant cell suspension culture technology has been hampered by the periodic fluctuations in metabolite levels, often observed in suspension cultures over multiple passages (Roberts 2007; Lee et al. 2010). Inconsistent cell culture product titers and flask to flask variations over passages can complicate the development of an economically viable cell culture bioprocess. Freezing and regenerating high-producing plant cell lines using cryopreservation techniques can alleviate such issues created during culture passage, and this approach has been successfully demonstrated for several plant species (Reinhold et al. 1995; Ishikawa et al. 2006). However, cryopreservation of plant cells is not a widely

*Corresponding author, sroberts@ecs.umass.edu, Phone: 413-545-1660, Fax: 413-545-1647.

established technique, because cryopreservation protocols and parameters such as pretreatment/preculture, freezing and post-thaw/regeneration have to be adapted and optimized for each plant species and cell line (Mustafa et al. 2011). Even if an optimized cryopreservation protocol exists for a species, post-thaw viability, growth and performance, and the issue of preserved cell lines differing from original ones hinder application of such methods (Boisson et al. 2012; Zeliang et al. 2010). Elicitation using abiotic or biotic compounds, such as jasmonic acid, is often used to enhance accumulation of secondary metabolites in suspension cultures (Gundlach et al. 1992; Suzuki et al. 2005; Pauwels et al. 2008; Krzyzanowska et al. 2012). Though elicitation increases culture metabolite yields, fluctuations in metabolite levels of *Taxus* cultures over multiple passages post-elicitation are observed (Kim et al. 2004).

Several studies in a variety of plant cell culture systems have reported fluctuations in secondary metabolite levels over culture passages (Callebaut et al. 1997; Qu et al. 2005; Hirasuna et al. 1991; Morris et al. 1989; Deusneumann and Zenk 1984). Genetic and epigenetic instabilities have generally been suggested as the primary causes for such variability (Qu et al. 2005; Zhao and Verpoorte 2007). Several other hypotheses have emerged to explain the observed variability including, development of heterogeneous populations in cell culture, amongst which only certain productive cells accumulate secondary metabolites (Hall and Yeoman 1986), changes in inter- and intracellular communication amongst cells (Hall and Yeoman 1987), and the influence of environmental factors (e.g., light, temperature, pH) or process parameters (e.g., oxygen levels, agitation) (Yeoman and Yeoman 1996; Saito and Mizukami 2002). Variability in secondary metabolite yield may also be created by inadequate control of factors such as initial cell density and time of inoculation during routine culture passage (Morris et al. 1989; Kolewe et al. 2008). Understanding the factors that influence fluctuations in secondary metabolite levels can aid in the design of improved bioprocessing strategies.

The tendency of plant cells to remain connected via cell walls and form aggregates has a considerable effect on bioprocess performance, including growth and metabolite levels (Patil et al. 2012; Capataz-Tafur et al. 2011). *Taxus* cell suspension cultures, which produce the valuable secondary metabolite paclitaxel, consist of aggregates ranging from 100 μm to over 2000 μm (Kolewe et al. 2010) (Fig.1). Recent studies from our laboratory indicate that the degree of cellular aggregation in *Taxus* cultures affects the level of paclitaxel accumulation (Kolewe et al. 2011). These results demonstrated the relationship between aggregate size and paclitaxel accumulation within individual experiments (Kolewe et al. 2011, Patil et al. 2012), but did not address long-term culture passage. Because plant cell cultures are typically maintained by non-selective culture passage over extended periods of time, an explicit relationship between aggregation patterns and corresponding secondary metabolite levels over multiple passages is important and can be used to suggest strategies for superior culture performance.

Inconsistencies during the culture passage procedure can cause changes in growth rate or affect the lag period such that cells in the next passage may be different metabolically with respect to both growth and metabolite production (Morris et al. 1989). Monitoring cell cycle distribution provides a useful means to understand the cell division potential of the cultures (Yanpaisan et al. 1999). Cultures with a high percentage of G2 phase cells have higher frequency of cell division. Secondary metabolite synthesis is often associated with differentiation of plant cells, which occurs after arrest of cells at the G1 or G2 phase (Bergounioux et al. 1992). Thus, monitoring cell cycle distributions over time can provide information on differentiation characteristics of the cultures. Instability of nuclear DNA content, which is often observed in terms of induced polyploidy or aneuploidy, is frequently observed in plant cell suspensions (Baebler et al. 2005; Creemers-Molenaar et al. 1992).

Variable ploidy levels have also been shown to affect secondary metabolite production in seedlings of *Hypericum perforatum* (Kosuth et al. 2003), hairy root cultures of *Artemisia annua* (De Jesus-Gonzalez and Weathers 2003) and *Hyoscyamus muticus* (Dehghan et al. 2012). Studies on genomic stability of *Taxus* cell cultures immediately following culture initiation (two-year timeframe) have been reported (Baebler et al. 2005) and indicate heterogeneity in genomic stability amongst cell lines; however, paclitaxel levels have not been correlated to nuclear DNA content or ploidy levels in well-established *Taxus* suspension cultures.

Here, the variation in aggregate size, extracellular sugar concentration, ploidy, and proportion of cells in particular phases of the cell cycle were quantified and correlated with paclitaxel accumulation over multiple passages (six months). Factors such as media composition, inoculation density, day of culture passage and cultivation temperature, which are known to influence secondary metabolite levels (Zhong et al. 1995; Qu et al. 2006), were kept constant. Determining factors that correlate with variable paclitaxel yields over long-term passage can lead to the development of new bioprocessing strategies to favorably control paclitaxel accumulation in *Taxus* suspension cell cultures.

Materials and methods

Cell culture maintenance, elicitation and biomass measurements

Taxus cuspidata cell line P93AF was provided by the U.S. Plant Soil and Nutrition Laboratory in Ithaca, NY, and maintained in our laboratory, as described previously (Naill and Roberts 2004). Suspensions were maintained in 250 mL Erlenmeyer flasks capped with Bellco (Vineland, NJ) foam closures at 23 °C and 125 RPM in gyratory shakers in the dark. For each culture passage, six replicate cultures were generated. Three cultures were sacrificed for analyses and three cultures were passaged to six new flasks for the next cycle. Culture passages were performed by transferring 20 mL of inocula (corresponding to a packed cell volume of 4–5 mL) originating from a 14-day old suspension culture into 80 mL of fresh medium. A Multisizer 3™ Coulter counter equipped with a 2,000 µm aperture (Beckman Coulter, Brea, CA) was used to measure biomass and culture aggregate size distributions, as described previously (Kolewe et al. 2010). For Coulter counter analysis, two × 2 mL samples of well mixed culture broth from each of the three replicate flasks were collected on day 7. Samples for extracellular sucrose and glucose analyses and nuclei isolation were also taken on day 7 (described below). Post-sampling, cultures were treated with 150 M methyl jasmonate (MeJA), as described previously (Naill and Roberts 2004). Following MeJA elicitation, 1 mL samples of well mixed culture broth containing both cells and media were taken on day 7 post-elicitation (day 14 in the cell culture passage) with a cut pipette tip and stored at –80 °C prior to taxane analysis (described below).

Glucose and sucrose measurements

The levels of extracellular sucrose and glucose were measured in cell culture media samples using a blood glucose analyzer (YSI 2700 Select Biochemistry Analyzer, YSI Life Sciences, Yellow Springs, OH). For analysis, 500 µL of cell culture media was collected on day 7 posttransfer. Briefly, dextrose (D-glucose) diffuses across a membrane (that contains glucose oxidase) in the analyzer. This reaction oxidizes the dextrose to hydrogen peroxide and D-glucono-δ-lactone. The hydrogen peroxide is amperometrically detected at a platinum electrode surface. The current produced is directly proportional to the hydrogen peroxide and dextrose concentrations in the sample. Sucrose is indirectly measured through enzymatic hydrolysis.

Isolation of intact nuclei for ploidy and nuclear DNA content analyses

Approximately 3–4 mL of well mixed culture broth (corresponding to 0.5 g wet biomass) was filtered over Miracloth (Calbiochem, CA) and used for isolation of intact nuclei (Gaurav et. al 2010). Samples were taken after every two passages. Briefly, one mL of Galbraith buffer (45 mM MgCl₂, 30 mM sodium citrate, 20 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), 0.5 % (w/v) Triton X-100, pH 7.0) at 4 °C was added to the biomass sample on a petri dish (50 mm × 12 mm), and the biomass was chopped with a sharp razor approximately 500 times to disrupt cell walls and allow for the release of intact nuclei. An additional 2 mL of Galbraith buffer was added to resuspend the chopped biomass, and this suspension was successively filtered over 80 µm and 30 µm nylon mesh (SmallParts, Inc., Miramar, FL).

For ploidy and cell cycle analysis, 50 l of 1 mg/mL RNase and 50 l of 1 mg/mL propidium iodide were added to 1 mL of the filtered nuclei solution. Samples were stained for 30–45 min. on ice before flow cytometric analysis (Becton Dickinson (San Jose, CA) LSRII analytical flow cytometer). Forward scatter and side scatter were collected on a logarithmic scale, and PI fluorescence was collected on a linear scale. A minimum of 5000 events was collected in the gated region of a forward scatter and side scatter plot corresponding to nuclei. For cell cycle analysis, Watson Pragmatic Model of FlowJo (v7.6) software (Tree Star, Inc.) was applied to the PI histogram to determine the percentage of cells in G0/G1, S and G2 phases. For nuclear DNA content analysis, 1 mL of the filtered nuclei solution was aliquoted and approximately ten thousand chicken erythrocyte nuclei singlets (Biosure, Grass valley, CA) were added as an internal standard. The mixture of *Taxus* nuclei and chicken nuclei was incubated with 50 l of 1 mg/mL RNase and 50 µl of 1 mg/mL PI for 30 minutes on ice, before flow cytometric analysis, as described above, where forward scatter, side scatter and PI fluorescence were collected on a logarithmic scale.

Taxane analysis

Taxanes were identified and quantified using a Waters Acquity UPLC H-Class system. Separation on UPLC was performed using an Acquity (Waters, Milford, MA) BEH C₁₈ column (particle size 1.7 µm, 50 mm × 2.1 mm). Samples were prepared for metabolite analysis, as described elsewhere (Naill and Roberts 2004). Paclitaxel and baccatin III authentic standards (Sigma-Aldrich, St. Louis, MO) were used to generate standard curves for quantification of metabolite content, and as a reference for comparison of sample peak retention times and characteristic taxane UV absorption spectra.

Results and Discussion

Relationship between paclitaxel levels and mean aggregate size

Paclitaxel levels measured in each culture passage (on day 7 post-elicitation with MeJa, day 14 of the cell culture period) varied over the six-month experimental timeframe, showing a 6.9-fold difference between the highest and lowest levels of accumulation (Fig. 2a). Such batch-to-batch variations can have significant economic implications in large scale fermentation systems. As the mean size of the aggregates was shown to affect the level of paclitaxel accumulated in cultures in distinct experiments (Kolewe et al. 2011), we measured the aggregate size distribution during each passage (Fig. 2a). Similar to paclitaxel levels in the culture, a saw-tooth pattern was observed for mean aggregate size when plotted against time (passage number). Both paclitaxel level and mean aggregate size in the cultures followed a distinct relationship: as the mean aggregate size of the culture decreased, the amount of paclitaxel accumulated in that culture passage increased. A significant negative correlation ($r = 0.75$, $p < 0.01$) was observed between mean aggregate size and paclitaxel accumulation (Fig. 2b). These results were consistent with previous observations (Kolewe et

al. 2011), where in individual experiments, cultures initiated with smaller aggregate size distributions accumulated higher paclitaxel levels than their larger aggregate counterparts. However, the differences in paclitaxel levels observed in this study were over sequential multiple passages without any manipulation of aggregate sizes prior to inoculation. Interestingly, similar variations in mean aggregate size were observed for cultures that do not accumulate paclitaxel post-elicitation (data not shown),

Since plant cell suspension cultures are inherently heterogeneous, it is not surprising that metabolite levels over time are not consistent. It is possible that unconscious selection of aggregates of a particular size class may occur during subculturing (Kinnersley and Dougall 1980), which in this case could contribute to the observed fluctuations in mean aggregate size, and hence paclitaxel yields. One of the reasons for inconsistent selection of aggregates of a particular size class could be due to the density difference between large and small aggregates present in the culture. Routine transfer of aggregated plant cells is performed by continuous gentle shaking of the parent flask while pipetting a desired volume of culture into fresh media using a wide-mouthed pipet. The tendency of large aggregates to sink faster could cause them to escape the pipette, potentially leading to unintentional exclusion of larger aggregates. In the case of suspension cultures where key secondary metabolites are pigments, the highly pigmented cultures are chosen via simple visualization for passage, which could lead to prolonged higher productivity (Yamada and Hashimoto 1990; Yeoman and Yeoman 1996). However, in suspension cell systems such as *Taxus*, secondary metabolites are not fluorescent or colored, preventing screening and selective subculturing via simple visualization. The data presented here suggest that selection of aggregates of a particular size class during subculturing could be a simple method for plant suspension systems such as *Taxus* to maintain high and stable producing cell lines.

Relationship between sucrose consumption, paclitaxel levels and mean aggregate size

There may be differential utilization of sugars from the media amongst culture passages, which could influence growth, aggregate size distribution, and ultimately paclitaxel accumulation. During each passage, the concentration of both glucose and sucrose was measured in the extracellular medium on day 7 post-inoculation. Extracellular sugar concentration ranged from 8.5 to 5.6 g/L on day 7 (initial value on day 0 is 20 g/L) and did not correlate with either paclitaxel level or culture mean aggregate size (data not shown). Thus, by day 7, cells in each passage utilized similar levels of sugar, irrespective of the mean aggregate size of the culture. This result was not completely unexpected as a similar amount of inocula was used to initiate cultures in each passage to avoid the effect of varying inoculation density on metabolite levels (Morris 1986). This result also suggests that for the range of aggregate sizes in these *Taxus* cultures, diffusion limitations for simple sugars are likely not present, as sugar consumption normalized by biomass was found to be independent of aggregate size.

Cell cycle, DNA content and ploidy analyses

Flow cytometry offers a rapid and accurate method for determining ploidy content, assessing DNA content and analyzing cell cycle participation. Monitoring the pattern of cell cycle distribution over culture passages provides information on cell division potential and differentiation characteristics at each passage (Neumann et al. 2009; Yanpaisan et al. 1999). For example, an increased proportion of cells in G0/G1 phase would indicate potential differentiation of cells into organized tissues (Yanpaisan et al. 1999). To examine the cell cycle activity for *Taxus* cells during each passage, the proportion of cells in each phase of the cell cycle, G0/G1, S and G2 were determined using flow cytometry. A similar percentage of cells remained in each cell cycle phase on day 7 of each passage (Fig. 3). Throughout the study, on day 7 of each passage, 72–78% cells were found in G0/G1-phase,

14–17% cells in S-phase, and 7–11% cells in G2-phase. These results suggest that prior to MeJA elicitation, no cell cycle inhibition or arrest occurs with repeated culture passage. Nonetheless, it is important to realize that this analysis was uni-variate, and does not distinguish between non-cycling G0 cells and cycling G1 cells, as both have 2C DNA content. A multi-parametric analysis with a cellular marker such as RNA (Bergounioux et al. 1988) or protein content (Citterio et al. 1992), or using thymidine analogs such as BrDU (Yanpaisan et al. 1998) or EdU (Kotogany et al. 2010), must be used to distinguish between cycling and non-cycling cells. Previous data illustrate that a significant portion of *Taxus* cells (~65 %) reside in G0 phase (Naill and Roberts 2005).

Nuclear DNA content correlates with ploidy level, and its estimation in relative units can be used to detect changes in ploidy levels amongst passages (Dolezel et al. 2007). Using chicken nuclei as a standard (2C DNA content = 2.33 picograms (pg)), the 2C DNA content of the *Taxus* P93AF cell line measured with flow cytometry was found to be ~ 36 pg (Fig. 4a). The nuclear DNA content measured on day 7 (mid-exponential phase) did not vary from one passage to another. Previous work has shown heterogeneity in nuclear DNA content amongst cell lines over a two-year timeframe following culture initiation, where some cell lines exhibit stable genome size (four of nine evaluated) and others (five of nine evaluated) show variation. (Baebler et al. 2005). During each passage, only two peaks were observed, corresponding most likely to 2C and 4C DNA (G1 and G2) content (Fig. 4b). Thus, no changes in ploidy and nuclear DNA content were detected throughout the six-month timeframe investigated here, implying orderly progression through mitosis. Changes in ploidy levels are often observed once suspension cultures are established (Maciejewska et al. 1999; Creemers-Molenaar et al. 1992), and in most cases an increased ploidy is seen as the culture ages. Varying the concentration of growth hormones has also been shown to induce changes in culture ploidy levels (Mishiba et al. 2001). However, the cell line examined here was maintained with the same concentration of growth hormones over time (Gibson et al. 1993). Constant ploidy levels and nuclear DNA content over the six-month timeframe indicate that ploidy levels in our mature *Taxus* cultures do not vary on the same time scale as metabolite production patterns, and are most likely not a cause of this relatively short term variability in yield. Other genetic factors such as structural changes in nuclear DNA, gene mutations and translocation of chromosomes to new segments, and/or epigenetic factors such as gene silencing by DNA methylation, may contribute to the observed differences in paclitaxel accumulation amongst passages, and require further study.

Conclusions

Uncertainty in product levels and limited success of plant cell cryopreservation techniques necessitates studies into understanding the reasons for variability in secondary metabolite accumulation in plant suspension cultures. *Taxus* cell cultures consist of a heterogeneous population of cells, with aggregates of varying sizes present in the culture. Here, we have shown that culture mean aggregate size is an important process parameter that correlates with variable paclitaxel accumulation during long term suspension culture maintenance. Sugar utilization, nuclear DNA content (i.e., ploidy levels) and cell cycle participation did not differ significantly amongst passages. Information regarding aggregation size distributions during a batch culture could be incorporated into kinetic models to more accurately predict culture growth, metabolism and product formation (Kolewe et al. 2012). This study further emphasizes the importance of rational manipulation of aggregate sizes during routine culture passage for optimization of plant cell culture bioprocesses (Hanagata et al. 1993; Kinnersley and Dougall 1980; Kolewe et al. 2011). This is particularly relevant in suspension systems where major secondary metabolite products are often not pigmented, and a simple visual selection of high metabolite producing cells is not always possible.

Acknowledgments

This work was supported by grants from the NIH (GM070852) and NSF (CBET-0730779). M.E.K. also acknowledges support from a National Research Service Award T32 GM08515 from the NIH and the NSF-sponsored Institute for Cellular Engineering IGERT Program (DGE-0654128). We thank Dr. Donna Gibson of the USDA, Agricultural Research Service, for the *Taxus* cell cultures.

References

- Baebler S, Hren M, Camloh M, Ravnikaar M, Bohanec B, Plaper I, Uzman R, Zel J. Establishment of cell suspension cultures of yew (*Taxus x media rehd.*) and assessment of their genomic stability. In *Vitro Cell Dev Biol Plant*. 2005; 41:338–343.
- Bergounioux C, Brown SC, Petit PX. Flow-cytometry and plant protoplast cell biology. *Physiol Plantarum*. 1992; 85:374–386.
- Bergounioux C, Perennes C, Brown SC, Gadal P. Nuclear-rna quantification in protoplast cell-cycle phases. *Cytometry*. 1988; 9:84–87. [PubMed: 2457475]
- Boisson AM, Gout E, Bligny R, Rivasseau C. A simple and efficient method for the long-term preservation of plant cell suspension cultures. *Plant Methods*. 2012; 8:4. [PubMed: 22289515]
- Callebaut A, Terahara N, Haan M, Decleire M. Stability of anthocyanin composition in *Ajuga reptans* callus and cell suspension cultures. *Plant Cell Tiss Org*. 1997; 50:195–201.
- Capataz-Tafur J, Trejo-Tapia G, Rodríguez-Monroy M, Sepúlveda-Jimenez G. Arabinogalactan proteins are involved in cell aggregation of cell suspension cultures of *Beta vulgaris L.* *Plant Cell Tiss Org*. 2011; 106:169–177.
- Citterio S, Sgorbati S, Levi M, Colombo BM, Sparvoli E. Pcn and total nuclear-protein content as markers of cell-proliferation in pea tissue. *J Cell Sci*. 1992; 102:71–78.
- Cragg GM, Grothaus PG, Newman DJ. Impact of natural products on developing new anti-cancer agents. *Chem Rev*. 2009; 109:3012–3043. [PubMed: 19422222]
- Creemers-Molenaar J, Loeffen JPM, van Rossum M, Colijn-Hooymans CM. The effect of genotype, cold storage and ploidy level on the morphogenic response of perennial ryegrass (*Lolium perenne L.*) suspension cultures. *Plant Sci*. 1992; 83:87–94.
- Dehghan E, Hakkinen ST, Oksman-Caldentey KM, Ahmadi FS. Production of tropane alkaloids in diploid and tetraploid plants and in vitro hairy root cultures of Egyptian henbane (*Hyoscyamus muticus L.*). *Plant Cell Tiss Org*. 2012; 110:35–44.
- Deusneumann B, Zenk MH. Instability of indole alkaloid production in *Catharanthus roseus* cell suspension cultures. *Planta Med*. 1984; 50:427–431. [PubMed: 17340345]
- Dolezel J, Greilhuber J, Suda J. Estimation of nuclear DNA content in plants using flow cytometry. *Nat Protocols*. 2007; 2:2233–2244.
- Eibl, R.; Eibl, D. Bioreactors for plant cell and tissue cultures. In: Oksman-Caldentey; Kirs-Marja; Barz, W., editors. *Plant biotechnology and transgenic plants*. New York: Marcel Dekker; 2002. p. 163-199.
- Gaurav, V.; Kolewe, ME.; Roberts, SC. Fett-Neto, A. *Plant Secondary Metabolism Engineering: Methods and Applications, Methods in Molecular Biology*. New York: Springer; 2010. Flow cytometric methods to investigate culture heterogeneities for plant metabolic engineering; p. 243-262.
- Gibson D, Ketchum R, Vance N, Christen A. Initiation and growth of cell lines of *Taxus brevifolia* (Pacific yew). *Plant Cell Rep*. 1993; 12:479–482.
- Gundlach H, Muller MJ, Kutschan TM, Zenk MH. Jasmonic acid is a signal transducer in elicitor induced plant cell cultures. *Proc Natl Acad Sci U S A*. 1992; 89:2389–2393. [PubMed: 11607285]
- Hall RD, Yeoman MM. Factors determining anthocyanin yield in cell cultures of *Catharanthus roseus (L.) G.Don*. *New Phytol*. 1986; 103:33–43.
- Hall RD, Yeoman MM. Intercellular and intercultural heterogeneity in secondary metabolite accumulation in cultures of *Catharanthus roseus* following cell line selection. *J Exp Bot*. 1987; 38:1391–1398.

- Hanagata N, Ito A, Uehara H, Asari F, Takeuchi T, Karube I. Behavior of cell aggregate of *Carthamus tinctorius* L cultured cells and correlation with red pigment formation. J Biotechnol. 1993; 30:259–269.
- Harvey AL. Natural products in drug discovery. Drug Discov Today. 2008; 13(19–20):894–901. [PubMed: 18691670]
- Hirasuna TJ, Shuler ML, Lackney VK, Spanswick RM. Enhanced anthocyanin production in grape cell cultures. Plant Sci. 1991; 78:107–120.
- Ishikawa M, Suzuki M, Nakamura T, Kishimoto T, Robertson AJ, Gusta LV. Effect of growth phase on survival of bromegrass suspension cells following cryopreservation and abiotic stresses. Ann Bot-London. 2006; 97:453–459.
- Kim BJ, Gibson DM, Shuler ML. Effect of subculture and elicitation on instability of Taxol production in *Taxus* sp suspension cultures. Biotechnol Progr. 2004; 20:1666–1673.
- Kinnersley AM, Dougall DK. Increase in anthocyanin yield from wild carrot cell cultures by a selection system based on cell aggregate size. Planta. 1980; 149:200–204.
- Kolewe ME, Gaurav V, Roberts SC. Pharmaceutically active natural product synthesis and supply via plant cell culture technology. Mol Pharm. 2008; 5:243–256. [PubMed: 18393488]
- Kolewe ME, Henson MA, Roberts SC. Characterization of aggregate size in *Taxus* suspension cell culture. Plant Cell Rep. 2010; 29:485–494. [PubMed: 20217417]
- Kolewe ME, Henson MA, Roberts SC. Analysis of aggregate size as a process variable affecting paclitaxel accumulation in *Taxus* suspension cultures. Biotechnol Progr. 2011; 27:1365–1372.
- Kolewe ME, Roberts SC, Henson MA. A population balance equation model of aggregation dynamics in *Taxus* suspension cell cultures. Biotechnol Bioeng. 2012; 109:472–482. [PubMed: 21910121]
- Kotogany E, Dudits D, Horvath GV, Ayaydin F. A rapid and robust assay for detection of S-phase cell cycle progression in plant cells and tissues by using ethynyl deoxyuridine. Plant Methods. 2010; 6:5. [PubMed: 20181034]
- Krzyzanowska J, Czubacka A, Pecio L, Przybys M, Doroszevska T, Stochmal A, Oleszek W. The effects of jasmonic acid and methyl jasmonate on rosmarinic acid production in *Mentha x piperita* cell suspension cultures. Plant Cell Tiss Org. 2012; 108:73–81.
- Lee EK, Jin YW, Park JH, Yoo YM, Hong SM, Amir R, Yan Z, Kwon E, Elfick A, Tomlinson S, Halbritter F, Waibel T, Yun BW, Loake GJ. Cultured cambial meristematic cells as a source of plant natural products. Nat biotech. 2010; 28:1213–1217.
- McChesney JD, Venkataraman SK, Henri JT. Plant natural products: Back to the future or into extinction? Phytochemistry. 2007; 68:2015–2022. [PubMed: 17574638]
- Mishiba KI, Okamoto T, Mii M. Increasing ploidy level in cell suspension cultures of *Doritaenopsis* by exogenous application of 2,4-dichlorophenoxyacetic acid. Physiol Plantarum. 2001; 112:142–148.
- Morris, P. Long term stability of alkaloid productivity in cell suspension cultures of *Catharanthus roseus*. In: Morris, P.; Scragg, AH.; Stafford, A.; Fowler, MW., editors. Secondary metabolism in plant cell cultures. Cambridge: University Press; 1986. p. 257–262.
- Morris P, Rudge K, Cresswell R, Fowler MW. Regulation of product synthesis in cell cultures of *Catharanthus roseus*. Plant Cell Tiss Org. 1989; 17:79–90.
- Mustafa NR, de Winter W, van Iren F, Verpoorte R. Initiation, growth and cryopreservation of plant cell suspension cultures. Nat Protocols. 2011; 6:715–742.
- Naill MC, Roberts SC. Preparation of single cells from aggregated *Taxus* suspension cultures for population analysis. Biotechnol Bioeng. 2004; 86:817–826. [PubMed: 15162458]
- Naill MC, Roberts SC. Cell cycle analysis of *Taxus* suspension cultures at the single cell level as an indicator of culture heterogeneity. Biotechnol Bioeng. 2005; 90:491–500. [PubMed: 15812805]
- Neumann, KH.; Kumar, A.; Imani, J. Cell Division, Cell Growth, Cell Differentiation. In: Neumann, KH.; Kumar, A.; Imani, J., editors. Plant Cell and Tissue Culture - A Tool in Biotechnology. Springer Berlin Heidelberg: Basics and Application; 2009. p. 235–247.
- Patil RA, Kolewe ME, Normanly J, Walker EL, Roberts SC. Contribution of taxane biosynthetic pathway gene expression to observed variability in paclitaxel accumulation in *Taxus* suspension cultures. Biotechnol J. 2012; 7:418–427. [PubMed: 22095859]

- Pauwels L, Morreel K, Witte ED, Lammertyn F, Montagu MV, Boerjan W, Inzé D, Goossens A. Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured *Arabidopsis* cells. *Proc Natl Acad Sci U S A*. 2008; 105:1380–1385. [PubMed: 18216250]
- Qu JG, Zhang W, Hu QL, Jin MF. Impact of subculture cycles and inoculum sizes on suspension cultures of *Vitis vinifera*. *Chin J Biotechnol*. 2006; 22:984–989.
- Qu JG, Zhang W, Yu XJ, Jin MF. Instability of anthocyanin accumulation in *Vitis vinifera* L. *va. Gamay Freaux* suspension cultures. *Biotechnol Bioproc E*. 2005; 10:155–161.
- Reinhold PJ, Schrijnemakers EWM, Iren F, Kijne JW. Vitrification and a heat-shock treatment improve cryopreservation of tobacco cell suspensions compared to two-step freezing. *Plant Cell Tiss Org*. 1995; 42:261–267.
- Roberts SC. Production and engineering of terpenoids in plant cell culture. *Nat Chem Biol*. 2007; 3:387–395. [PubMed: 17576426]
- Saito, K.; Mizukami, H. Plant cell cultures as producers of secondary compounds. In: Oksman-Caldentey; Kirsi-Marja; Barz, W., editors. *Plant Biotechnology and Transgenic Plants*. Marcel Dekker; New York: 2002. p. 66-90.
- Suzuki H, Reddy MSS, Naoumkina M, Aziz N, May GD, Huhman DV, Sumner LW, Blount JW, Mendes P, Dixon RA. Methyl jasmonate and yeast elicitor induce differential transcriptional and metabolic re-programming in cell suspension cultures of the model legume *Medicago truncatula*. *Planta*. 2005; 220:696–707. [PubMed: 15605242]
- Yamada, Y.; Hashimoto, T. Possibilities for improving yield of secondary metabolites in plant cell cultures. In: Nijkamp, HJ.; Van der Plas, L.; Van Aartrijk, J., editors. *Current Plant Science and Biotechnology in Agriculture. Progress in Plant Cellular and Molecular Biology*. The Netherlands: Kluwer; 1990. p. 547-556.
- Yanpaisan W, King NJC, Doran PM. Analysis of cell cycle activity and population dynamics in heterogeneous plant cell suspensions using flow cytometry. *Biotechnol Bioeng*. 1998; 58:515–528. [PubMed: 10099288]
- Yanpaisan W, King NJC, Doran PM. Flow cytometry of plant cells with applications in large-scale bioprocessing. *Biotechnol Adv*. 1999; 17:3–27. [PubMed: 14538141]
- Yeoman MM, Yeoman CL. Manipulating secondary metabolism in cultured plant cells. *New Phytol*. 1996; 134:553–569.
- Zeliang PK, Pattanayak A, Iangrai B, Khongwir EA, Sarma BK. Fertile plant regeneration from cryopreserved calli of *Oryza rufipogon* Griff. and assessment of variation in the progeny of regenerated plants. *Plant Cell Rep*. 2010; 29:1423–1433. [PubMed: 20960202]
- Zhao J, Verpoorte R. Manipulating indole alkaloid production by *Catharanthus roseus* cell cultures in bioreactors: from biochemical processing to metabolic engineering. *Phytochem Rev*. 2007; 6:435–457.
- Zhong J, Yoshida M, Yoshida T. Effects of biological factors on cell growth and anthocyanin formation by suspended cultures of *Perilla frutescens* cells. *Chin J Biotechnol*. 1995; 11:143–147. [PubMed: 8562851]

Fig. 1. Characteristic aggregates observed in *T. cuspidata* P93AF cell suspension cultures. Arrow indicates typical morphology of cellular debris.

Fig. 2.

a. Fluctuations in mean aggregate size and paclitaxel levels over multiple passages of *T. cuspidata* P93AF cell suspension cultures. Passage is done every 14 days. Squares represent the mean aggregate size of the culture obtained through Coulter counter measurements.

Circles represent paclitaxel content in the culture as measured through UPLC. **b.**

Relationship between culture mean aggregate size and paclitaxel level. The Pearson correlation coefficient (r) is -0.75 ($p < 0.05$ as determined using a two-tailed test), indicating a statistically significant negative linear relationship. Data points represent three biological replicates. Horizontal bars represent standard errors of the mean aggregate size and vertical bars represent standard errors of the culture paclitaxel level.

Fig. 3. Cell cycle analysis of *T. cuspidata* P93AF cell line over multiple passages. The Watson Pragmatic Model of FlowJo (v7.6) software (Tree Star, Inc.) was applied to the propidium iodide histograms to differentiate cell cycle phases.

Fig. 4.

Flow cytometric DNA histograms **a.** Semi-log plot of DNA content in chicken nuclei (CN) singlets (2.33 pg 2C DNA content) and *Taxus* nuclei (TN), stained with 50 g/ml propidium iodide. The first peak is for chicken nuclei singlets (CN) and the next two peaks are for *Taxus* nuclei (TN) **b.** Linear plot of DNA content for *Taxus* nuclei stained with 50 g/ml propidium iodide. Cell cycle phase is indicated on the figure. Coefficients of variation were below 4% for all measurements.