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Interest of cellular differentiation in the production of vincristine and vinblastine in suspension cultures of *Catharanthus roseus* (L.) G Don.

Interés de la diferenciación celular en la producción de vincristina y vinblastina en cultivos en suspensión de *Catharanthus roseus* (L.) G Don.

D.A. Zavala-Ortiz[1,2], M.J. Martínez-Montero[1], E. Guedon[2], A. Marc[2], B. Ebel[2], D.M. Barradas-Dermitz[1], P.M. Hayward-Jones[1], M. Mata-Rosas [3], M.G. Aguilar-Uscanga[1*]

[1] *Tecnológico Nacional de México/Instituto Tecnológico de Veracruz: Calz. M.A. de Quevedo 2779, Veracruz, Ver. México.*
[2] *Laboratoire Réactions et Génie des Procédés, UMR 7274, CNRS-Université de Lorraine, 2 avenue Foret de Haye, TSA 40602, 54518 Vandouvre-les-Nancy, France.*
[3] *Instituto de Ecología A.C.: Carretera antigua a Coatepec 351, Xalapa, México.*

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Abstract

Plant cell culture technology, particularly suspension cultures, appears as a convenient tool to industrially produce molecules such as the anticancer molecules vincristine and vinblastine. Cell differentiation is needed for their *in vivo* synthesis and thus *in vitro* cultures have usually been considered as limited producing platforms. Several studies have recently detected vincristine and vinblastine in early differentiated calluses and also in suspension cultures. Nevertheless, the degree of cell differentiation has not been addressed, particularly in suspension cultures that could be used as a large-scale producing platform. Therefore, the effect of culture conditions on the production of vincristine and vinblastine, taking into account cytodifferentiation within cell aggregates, has been analyzed for the first time. Culture conditions such as light exposure and plant growth regulator regimes have been shown to affect cell differentiation. Moreover, cell differentiation was observed to be closely related to vincristine and vinblastine titers. Results provide important clues into the comprehension of *in vitro* culture performance for metabolites production requiring *in vivo* cell differentiation. They demonstrated the utility of taking into account cell differentiation for the further development of novel advanced processes of differentiated cell suspension cultures for producing valuable molecules, including biological medicines such as vincristine and vinblastine.

Keywords: *Catharanthus roseus* suspension culture, cell differentiation, biological medicines, vincristine, vinblastine.

Resumen

La tecnología del cultivo de células vegetales, aparece como una herramienta conveniente para producir de forma industrial moléculas como los anticancerígenos vincristina y vinblastina. La diferenciación celular es necesaria para su síntesis *in vivo* y, por tanto, los cultivos *in vitro* se han considerado habitualmente como plataformas de producción limitadas. Varios estudios han detectado recientemente vincristina y vinblastina en callos y también en cultivos en suspensión. Sin embargo, no se ha abordado el grado de diferenciación celular, particularmente en cultivos en suspensión que podrían usarse como plataforma de producción a gran escala. Por lo tanto, se ha analizado por primera vez el efecto de las condiciones de cultivo sobre la producción de vincristina y vinblastina, teniendo en cuenta la citodiferenciación dentro de los agregados celulares. Se ha observado que las condiciones de cultivo afectan la diferenciación celular. Además, ésta estaba estrechamente relacionada con la presencia de vincristina y vinblastina. Los resultados proporcionan pistas importantes sobre la comprensión del cultivo *in vitro* para la producción de metabolitos que requieren diferenciación celular *in vivo* para el desarrollo posterior de nuevos procesos avanzados que permitan producir moléculas valiosas, incluidas medicinas biológicas como la vincristina y la vinblastina.

Palabras clave: Cultivo en suspensión de *Catharanthus roseus*, diferenciación celular, medicinas biológicas, vincristina, vinblastina.

* *Corresponding author. E-mail*: maguilaruscanga@yahoo.com.mx
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1 Introduction

Catharanthus roseus (L.) G. Don, Madagascar periwinkle, has been used in traditional medicine against several diseases such as malaria and diabetes. It is one of the most extensively investigated medicinal plants because of its capacity to produce high economic value phytochemicals such as the antineoplastic molecules vincristine (VC) and vinblastine (VB) (Qu, Safonova, & De Luca, 2019) and the antihypertensive ajmalicine (Tikhomiroff & Jolicoeur, 2002). VC and VB are dimer-indo-alkaloids with molecular weights of 825 and 811 $gmol^{-1}$ respectively. The difference between VB and VC is that the former has a methyl while the latter has a formyl group on the indole nitrogen of the vindoline skeleton. Due to the complexity and scarcity of VC and VB within plants ($\approx$ 0.00015 % DW), production is mainly carried out from the coupling of the precursors catharanthine and vindoline present in higher amounts in plant material (Alam, Naeem, Khan, & Uddin, 2017). Nowadays production is mainly carried out by extraction from plant material grown in the United States, Spain, China, Africa, Australia, India and Southern Europe (Barkat, Abul, & Rahman, 2017). However, expensive extraction procedures are required with very low recovery. Thus, intensive efforts have been invested for increasing availability such as production via *in vitro* cultures, particularly suspension cultures feasible for scale-up.

Production of phytochemicals using industrial scale suspension culture in bioreactors has been proven feasible but limited to a few molecules such as paclitaxel, shikonine, berberine, ginseng, among others (Bourgaud, Gravot, Milesi, & Gontier, 2001). Nonetheless, no commercial production using *C. roseus* suspension culture has been set in motion. Production of VC and VB in plants requires intracellular and intercellular translocation of pathway intermediates within differentiated cells in leaves (Murata & Luca, 2005; St-Pierre, Vazquez-Flota, & De Luca, 1999). This complex synthesis partially explains why attempts for producing VC and VB by cell culture technology have failed, particularly in undifferentiated cell suspension cultures (Verpoorte, van der Heijden, Schripsema, Hoge, & Ten Hoopen, 1993).

This former conception has been challenged by several studies demonstrating VC and VB production capability by *in vitro* cultures, particularly of calluses with early differentiation into roots or shoots (Ataei-Azimi, Hashemloian, Ebrahimzadeh, & Majd, 2018; Kalidass, Mohan, & Arjunan, 2010; Miura, Hirata, & Kurano, 1987). These reports have claimed that production capability is closely related to cell differentiation, though no further details are provided. Moreover, suspension cultures with VC and VB production capability have also been reported (Taha, Shams, Nazif, & Seif-El-Nasr, 2014; Zhang *et al*., 2015), although no inferential about this capacity was provided.

The conception that *in vitro* cultures are not capable for VC and VB production mainly relies on the fact that *in vivo* synthesis of the precursor vindoline has been localized in chloroplasts (De Luca & Cutler, 1987) and thus *in vitro* heterotrophic cultures lacking functional chloroplasts could not synthetize the precursor. Autotrophic suspension cultures of *Catharanthus roseus* cell lines have been developed seeking production in functional chloroplasts but neither vindoline, VC nor VB was detected (Tyler, Kurz, & Panchuk, 1986). On the other hand, there have been reports of vindoline producing cultures under heterotrophic conditions (Naaranlahti *et al*., 1989; Scott, Mizukami, Hirata, & Lee, 1980). Indeed, it was recently discovered that suspension cultures of cambial meristem cells contained complete sets of enzymes that are responsible for the production of VC and VB (Zhang *et al*., 2015) from vindoline. Thus, there must be alternative metabolic pathways in *in vitro* heterotrophic cultures for vindoline, VC and VB, which could be activated by cell differentiation. Therefore, this study sought to analyze cell differentiation in suspension cultures and its eventual relationship with VC and VB production capability. A broad spectrum of culture conditions based on plant growth regulators (PGR) regimens and photoperiods was tested for inducing different cell differentiation and production capabilities. This study was exploratory and interpretative in nature, seeking to be the starting point for further research which could help to understand the nature of VC and VB *in vitro* synthesis for novel development of differentiated cell suspension culture processes.

2 Materials and methods

2.1 Generation of suspension cultures

Callus lines were generated from a mature *C. roseus* plant at the Francisco Javier Clavijero Botanical Gardens (Instituto de Ecología, A.C., Xalapa, Ver. Mexico). Plant material passed through a sterilization procedure and then explants from stems, leaves, apical, intercalary and lateral meristem tissues were placed on MS (Murashige and Skoog) medium with 30 gL^{-1} sucrose, 6.5 gL^{-1} agar (Caisson, Micropropagation Powder Type I), 4.52 μM 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and 4.44 μM 6-Benzylaminopurine (BAP) in darkness. Subsequently 374 calluses were induced, multiplied and used for further experiments. Calluses were transferred to the Plant Cell Lines Bank of the Veracruz Institute of Technology and maintained under four different photoperiods. Calluses maintained in a 16 hour per day (h d^{-1}) photoperiod (photonic flux of 30 μmol m^{-2} s^{-1}) were used. Selected calluses with similar color and friability from stem, leaf and apical meristem tissues were aseptically transferred to 250 mL Erlenmeyer flasks containing 100 mL MS liquid medium supplemented with 2 mgL^{-1} glycine, 100 mg L^{-1} myo-inositol (Sigma-Aldrich), 30 g L^{-1} sucrose, 4.52 μM 2, 4-D, and 4.44 μM BAP (proliferation medium). The pH was adjusted to 5.7 ± 0.1 with 0.5 N NaOH and 0.5 N HCl and the medium was autoclaved at 121°C for 15 min. For subsequent subculturing in fresh medium, a volume of 10 mL of the original culture was inoculated into a 125 mL Erlenmeyer flask containing 40 mL proliferation medium every 15 days.

Once suspension culture was established, inocula for further experiments were always six-day-old biomass suspension cultures that were centrifuge-concentrated (5 min, 250 rcf, 15 ºC). Pellets were then re-suspended in agitated (magnetic stirrer, 90 rpm) fresh medium (≈ 50 mL) until a cell dry weight of approximately 3 $gL^{-1}$was achieved, then aliquots were taken to inoculate flasks for the various experiments. Initial biomass dry weight concentration for flask experiments was 0.06 gL^{-1}± 0.01.

2.2 Effect of plant growth regulators and photoperiods on leaf cell line

Suspension cultures were initiated from calluses as previously described, then propagated during 12 subcultures in proliferation medium. For evaluating the effect of culture conditions, culture medium was based on basal proliferation medium with specific PGR composition. Firstly, the effect of three single PGR was studied: 2, 4-D (4.5 μM), 1-Naphthaleneacetic acid (NAA) (4.5 μM) and Gibberellic acid (GA_3) (4.5 μM). Secondly, mixtures of 2, 4-D (4.5 μM) with citokines (Kinetine (Kin) and BAP) were tested: 2, 4-D + BAP (2.25 μM); 2, 4-D + BAP (4.5 μM); 2, 4-D + Kin (2.25 μM) and 2, 4-D + Kin (4.5 μM). One treatment without any exogenous PGR was included as a control, thus eight PGR regimens were tested.

The same inoculum, a previously 12-fold subcultivated suspension culture, was used for all tested treatments to reduce the effect of common plant cell heterogeneity associated to inoculum history. Flasks with silicone sponge closure (Chemglass, CLS-1490-038) were incubated for 25 days at room temperature with orbital agitation (0.22 rcf) in a rotary shaker (New Brunswick G70; 7 $\mu molm^{-2}s^{-1}$ photonic flux). Four different photoperiods: 0 h (total darkness), 8 hd^{-1}, 16 hd^{-1} and 24 hd^{-1} (continuous light exposure) were used. As four different photoperiods and eight different PGR regimens were tested, the impact of 32 operating conditions on alkaloid production and cell differentiation was evaluated.

2.3 Production of VC and VB by differentiated cell culture in a bioreactor

Two bioreactor batch cultures were used to analyze the dynamics of cell differentiation and VC and VB production in suspension cultures. Differentiated cell subpopulations were measured during the whole culture, as also were the concentrations of VC and VB. Cultures were carried out in a 3 L benchtop bioreactor (Applikon, the Netherlands) with a 2 L working volume using marine impeller performed agitation (90 rpm; 30° C). For the first culture, dissolved oxygen (DO) was controlled at 50 % air saturation, while for the second culture it was measured but not controlled. The bioreactor jars were illuminated in a 16 hd^{-1} photoperiod (50 $\mu mol.m^{-2}$ s^{-1} photonic flux). A leaf cell line already subcultured four times during a 6-day period was used as inoculum. The culture media was MS medium supplemented with 2, 4-D (4.5 μM) + BAP (4.5 μM) for both bioreactor cultures.

For the generation of inoculums, calluses with similar friability, color and age were used to generate suspension cultures. Approximately 5 g fresh callus was placed into 20 mL liquid MS media supplemented

with 2 mgL^{-1} glycine, 100 mgL^{-1} myo-inositol, 30 gL^{-1} sucrose, 4.5 μM 2, 4-D, 4.44 μM BAP. The pH was adjusted to 5.7 ± 0.1 with 0.5 N NaOH and 0.5 N HCl and the medium was autoclaved at 121 °C for 15 min. Cultures were then incubated during six days in 125 mL Erlenmeyer flasks in a 16 hd^{-1} photoperiod (30 $\mu molm^{-2}s^{-1}$ photonic flux) at 25 °C and shaken at 100 rpm on an orbital shaker. After the first culture, the suspension culture was filtered using a sterile stainless-steel mesh (2.25 mm^2 pore size) to retain callus clusters, then approximately 20 mL culture volumes were used to equally inoculate two flasks containing 10 mL culture media, which were subsequently cultured under the same conditions. Two further subcultures were performed for biomass propagation. Sixteen flasks containing a total of 320 mL were then concentrated by repeated cell decantation until an approximately 100 mL concentration was achieved which was used to inoculate bioreactor cultures. This procedure was repeated for every bioreactor inoculation.

2.4 Determination of biomass concentration and growth

Biomass growth was measured by gravimetric method (changes in dry weight). In a laminar flow cabinet, 1.0 mL suspension culture from a thoroughly stirred medium was sampled and put in an already weighed 1.5 mL micro-centrifuge tube. The tube was centrifuged (10,000 rcf, 10 min; Eppendorf 5424), its content decanted and then 1.0 mL deionised water added. Centrifugation and decantation were repeated and the washed pellet vacuum-dried (65 °C, ShellLab mod. 1410) until constant weight was reached, and then weighed (Mettler H80).

2.5 Cell differentiation analysis

Cell aggregates within cultures were histologically analyzed by optical microscopy (Motic, MO-567, USA) as previously reported (Zavala-Ortiz *et al.*, 2020). Differentiation analysis of cells in suspension cultures was undertaken based on cell wall differences; either thin primary-walled cells (TPWC), or thin slightly thickened primary-walled cells (TSTPWC) or secondary-walled cells with spiral thickening (SWCST) (Crang, Lyons-Sobaski, & Wise, 2018; Mauseth, 2014; Savidge, 1983) and all cells were counted. A sample of 1 mL was put into a 1.5 mL micro-centrifuge tube and centrifuged (250 rcf, 15 min); 900 μL of the supernatant was put in a tube and 900 μL digestion enzyme (TrypLE™ Express Enzyme (1X), Thermo Fisher Scientific) was added to the pellet. The tube was agitated in a vortex (MX-S, Science MED) for 45 min. It was centrifuged with the same conditions, and the pellet was isolated in 300 μL by discarding the supernatant, resulting in a digested cell suspension suitable for analysis. Cells in digested cell suspensions were analyzed and counted twice in a Neubauer improved chamber (Blaubrand), each count comprising between 105 and 180 single cells. Cell differentiation analysis was performed at the end of cultures in flask experiments and periodically in bioreactor cultures.

2.6 Determination of alkaloids, vincristine and vinblastine

Wagner's reagent was used for identification of alkaloids in cells (Giordano, Maleci, Agati, & Petruccelli, 2020; Mamoucha & Christodoulakis, 2016; Pratiwi, Sumaryono, Sari, & Ratnadewi, 2018; Yoder & Mahlberg, 1976); 100 μL digested cell suspension was mixed with 100 μL Wagner's reagent and incubated for 5 min. Cell observation was then carried out in a Neubauer improved chamber. Alkaloids within cells were detected by the characteristic reddish-brown color (Motic, MO-567, USA). Analysis of VC and VB in culture media (biomass with the medium) consisted of sample preparation according to Tikhomiroff and Jolicoeur, 2002 and Iskandar and Iriawati, 2016, with minimal differences. Volume samples (20-45 mL) were lyophilized for 24 h, $90x10^{-3}$ mbar, -43 °C (Labconco, Freezone 4.5, USA); the freeze-dried sample was weighed and approximately 0.100 g were taken for extraction with 1.0 mL HPLC grade methanol, using sonication for 1 h (Cole Parmer, CPX75, U.S.A). The sonicated extract was centrifuged (Eppendorf, 5415C, USA) at 12000 rcf, 10 min and, after filtration; the supernatant was transferred to an HPLC vial. Quantification was carried out by an isocratic reverse-phase HPLC procedure adaptation based on Gupta *et al*. (2005) and (Iskandar & Iriawati, 2016), where identity and homogeneity of peaks were checked by comparing photodiode array-generated data with VC and VB using library matching. Reference materials used for each calibration curve were Sigma-Aldrich VB (vinblastine sulfate V1377) and VC (vincristine sulfate V8879). Calibration curves were generated using 5 data points for both VC (0-100 gmL^{-1} concentration frame; 17.72 min retention time; 0.9848 R^2; 0.9924 R)

and VB (0-500 gmL^{-1}concentration frame; 31.53 min retention time; 0.9952 R^2, 0.9976 R). A Waters 600 HPLC System (Milford, Mass., U.S.A.) was used: a ChromolithR Performance RP-18 end-capped 100-4.6 HPLC column; acetonitrile-0.1M/ phosphate buffer mobile phase containing 0.5 % glacial acetic acid; pH 3.5 (21/79 v/v); 1.2 $mLmin^{-1}$ flow rate; Waters 2487 detector, λ 205 nm. Samples were compared against VC and VB external standard curves based on retention times.

2.7 *Statistics*

Data were analyzed with a one-way ANOVA and post hoc Tukey comparison tests ($p \leq 0.05$) using MATLAB environment (R2016a version; MathWorks Inc.).

3 Results

3.1 *Generation of suspension cultures*

The contamination from explants was controlled after two months by discarding contaminated tissues in three subcultures, from 374 initial tissue explants (Fig. 1 a), only 143 did not show apparent microorganism presence, internal microorganism contamination could not be rejected since clean-looking cultures were contaminated sporadically. The application of different plant growth regulators in the MS medium caused dedifferentiation on the tissues, it caused callus formation in the majority of the explants.

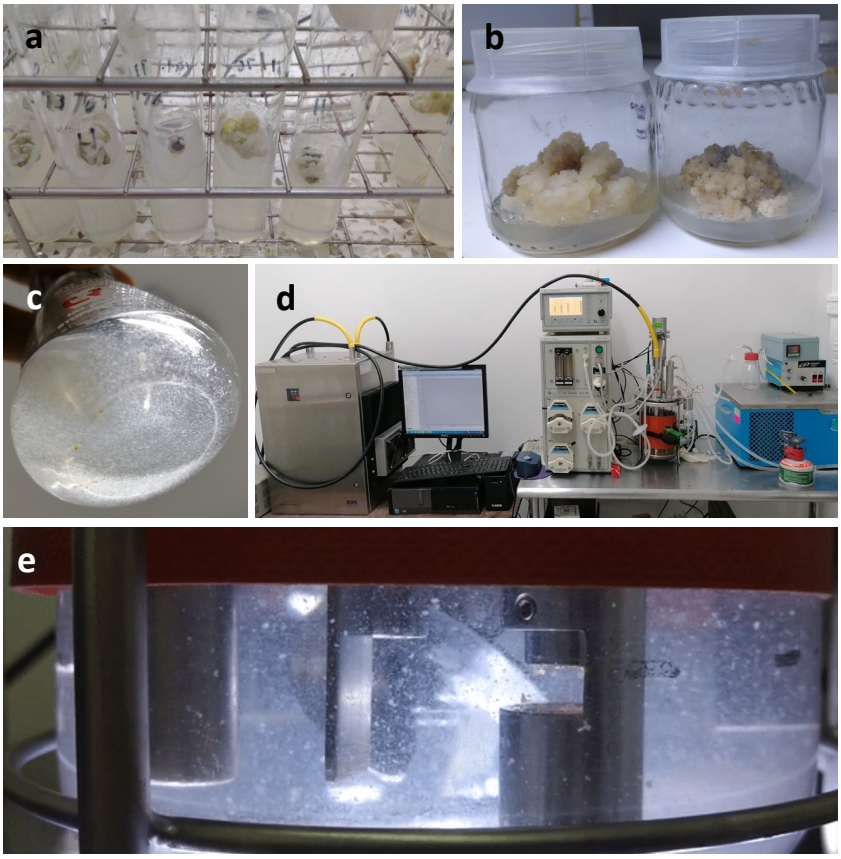

Fig. 1. Cells in culture systems. (a) Initiation of *C. roseus* callus cultures. (b) Typical aspect of well-established callus cultures just before subculture. (c) Typical aspect of well-established suspension cultures after inoculation during subcultures. (d) Bioreactor system used for the experiments. (e) Typical aspect of suspension cultures in bioreactors just after inoculation.

Table 1 Effect of photoperiods and PGR on VC and VB production in suspension cultures.

			Exogenous plant growth regulator regimens							
			No PGR	2,4-D (4.5 µM)	NAA (4.5 µM)	GA_3 (4.5 µM)	2,4-D (4.5 µM) BAP (2.25 µM)	2,4-D (4.5 µM) BAP (4.5 µM)	2,4-D (4.5 µM) Kin (2.25 µM)	2,4-D (4.5 µM) Kin (4.5 µM)
Light exposure photoperiod (hd^{-1})	0	VC	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
		VB	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	8	VC	1^{a}	19^{b}	0^{a}	3^{a}	2^{a}	1^{a}	3^{a}	2^{a}
		VB	1^{a}	1^{a}	0^{a}	21^{b}	0^{a}	0^{a}	17^{b}	13^{b}
	16	VC	0^{a}	34^{d}	0^{a}	0^{a}	23^{b}	27^{b}	25^{b}	30^{b}
		VB	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}
	24	VC	0^{a}	25^{c}	0^{a}	0^{a}	0^{a}	38^{c}	35^{c}	37^{c}
		VB	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}

2, 4-D: 2, 4-Dichlorophenoxyacetic acid, NAA: 1-naphthaleneacetic acid, BAP: 6-benzylaminopurine, Kin: Kinetin.
VC: Vincristine titer (mg/L), VB: vinblastine titer (mg/L), VC and VB analyzed at 25 d; superscript letters indicating statistical difference between groups due to the effect of light exposure in a single PGR regimen

When forming callus, callus induction time for stems, leaves, meristem and apical meristem tissues were almost the same range (14-20 d) for all treatments of plant growth regulators regimens. Establishment of clean cultures was successfully achieved (Fig. 1 b) for the further development of suspension culture lines. Cell aggregation was reduced with each subculture, negligible at the 4th subculture since the majority of aggregates freely passed through a needle (1.5 mm inner diameter), and therefore only suspension cultures with aggregates smaller than 1.5 mm were used for further experiments (Fig. 1 c). For subsequent experiments, only leaf cell line was selected for enhanced capabilities (data not shown). No important oxidation issues were observed neither in flasks (Fig. 1 c) nor in bioreactor (Fig. 1 e) cultures.

3.2 Effect of culture conditions in flasks

Changing culture media or operating conditions will always show a change in culture performance, though it usually takes 5-10 subcultures for final effects. Culture conditions affected cell production capacity in the first subculture as shown in Table 1 and detection of VC and VB varied differently depending on culture conditions. Light exposure had a strong effect since neither VC nor VB was detected in dark conditions independently of PGR regimen. Detection of VB only occurred when using an 8 hd^{-1} photoperiod. Evaluation of the effect of light exposure (photoperiod) in each individual PGR regimen revealed that there was no statistical difference (One-way ANOVA at 95 % confidence level) in VB titers either for cultures with NAA, 2,4-D as sole PGR, those including BAP or control culture (without exogenous PGR). Thus, the culture conditions that effectively induced VB production were those including GA_3 and mixtures of 2,4-D and Kin. As for VC titers, there was no statistical difference (One-way ANOVA at 95% confidence level) between photoperiods for treatments including NAA, GA_3, or control culture. Thus, the culture conditions that effectively induced VC production were those that contained 2,4-D.

3.3 Cell differentiation and its relationship with VC and VB production in flasks

As VC and VB were detected, a possible relationship between *C. roseus* cell differentiation in suspension cultures and VC and VB titers was then investigated, after first classifying differentiated cells, based on cell wall differences, as TPWC, TSTPWC and SWCST. Cell aggregates were observed, and their compositions analyzed seeking differentiated cells based on cell wall differences. During primary analysis, it was discovered that *C. roseus* aggregates contained several cell subpopulations in cell aggregates that achieved sizes up to 300 μm (Fig. 2 a), although smaller sizes were more common (Fig. 2 b). The three main types of differentiated cells were present in cell aggregates.

Examples of TPWC and TSTPWC are shown in Fig. 2 c, which also shows an example of cell differentiation from TPWC to TSTPWC status. Cell differentiation process to SWCST status is also shown in Fig. 2 d. The most common differentiated cell type

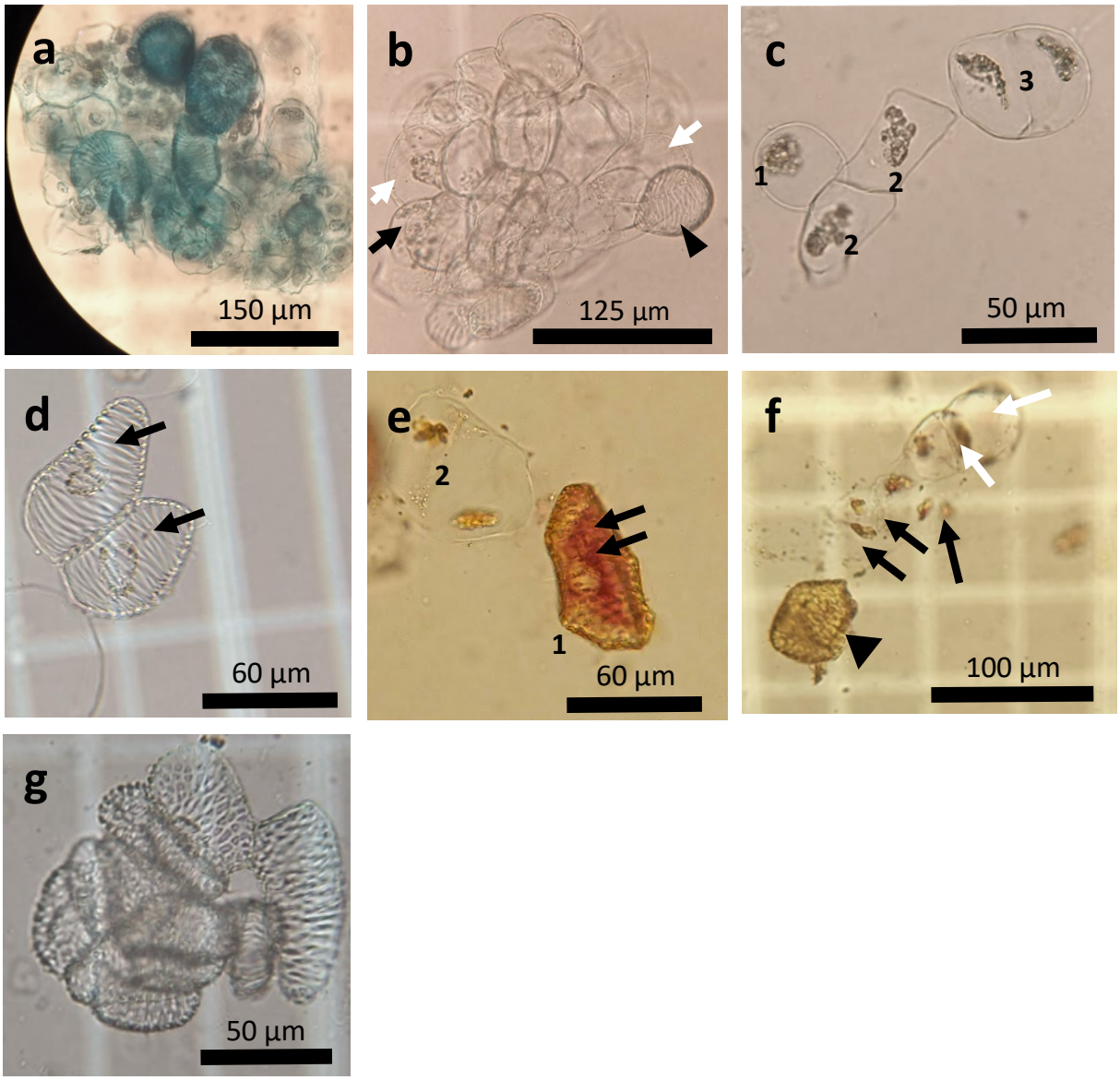

Fig. 2. Micrographs of differentiated cells during *C. roseus* suspension cultures. (a) Large aggregate with dead cells (SWCST) stained by Evans blue. (b) Typical size aggregate with differentiated cells: TPWC (white arrows), TSTPWC (black arrows) and a SWCST (triangle). (c) Example of a TPWC (1), TSTPWC (2) and the differentiation process into TSTPWC from TPWC status (3). (d) Example of differentiation process into SWCST (arrows). (e) A SWCST (1) stained reddish-dark brown, a positive reaction for alkaloids with Wagner's reagent (arrows) and TPWC (2) negative for alkaloids. (f) TPWC (dark arrows), TSTPWC (white arrows) and SWCST (triangles) negative for alkaloids with Wagner?s reagent. (g) Example of cell aggregate composed of only SWCST. (h) Bioreactor system used for the dynamic analysis of cell differentiation.

was TSTPWC, which usually comprised the majority of registered cells. TPWC were observed with nuclei and visible organelles and chlorenchyma-like cells to a lesser degree. The presence of differentiated cells within cell aggregates may imply that there could be some kind of early specialization or at least cells with different metabolic characteristics which could be related to enhanced alkaloid production. Therefore, Wagner's reagent was used for staining cells and revealing the presence of alkaloids within

cells (Giordano *et al*., 2020). TPWC were negative for the stain (Fig. 2 e2) while SWCST (Fig. 2 e1) were colored reddish-dark brown indicating the presence of alkaloids. The presence of differentiated cells with different alkaloid contents could explain differences in VC and VB titers (Table 1). This hypothesis was addressed by evaluating the relationship between differentiated cell population distributions and VC and VB titers. The inherent nature of suspension cultures to form aggregates was not suitable for direct cell differentiation state identification; therefore, to study cell differentiation, subpopulation distribution was measured by using single cell count in enzymatic digested suspension cultures.

A relationship between SWCST concentration and VC concentration was found when data from all culture conditions was plotted, with a relative high determination coefficient (R^2) of 0.88 (data not shown). From all the operating conditions tested, the culture treated with 2, 4-D (4.5 μM) plus BAP (4.5 μM) was selected because consistent data in terms of small standard deviations for VC concentration was observed. This led to significant differences between VC productions obtained with the different photoperiods tested (One-way ANOVA, post hoc Tukey, $p \leq 0.01$). Results were compared to cultures with NAA (4.5 μM) where no production of VC or VB was observed. Differentiated cell distribution for these two PGR regimens at four different photoperiods is given in Table 2. The culture with NAA (4.5 μM) contained low levels of SWCST independent of photoperiod used, while increased light exposure in the 2, 4-D (4.5 μM) plus BAP (4.5 μM) condition resulted mainly in SWCST differentiation, up to 28 % of total cell population during continuous light cultures. This increase in SWCST percentage was parallel to the increase in VC production (Table 2). These data strongly suggested that cell differentiation was responsible for different VC production capacity of the cultures. Composition of aggregates of NAA cultures was similar to the aggregate shown in Fig. 2 b, while aggregates composed of only SWCST (Fig. 2 g) were commonly observed in cultures with relatively high VC and VB titers.

3.4 Dynamics of cell differentiation in bioreactors

Results in flask cultures confirmed that culture conditions, such as addition of PGR and light exposure, strongly affected VC and VB titers; moreover, such production was related to cell differentiation, particularly to SWCST.

Table 2 Differentiated cell distribution in VC and VB producing and non-producing suspension cultures.

			Exogenous plant growth regulator regimens			
			NAA (4.5 µM)		2,4-D (4.5 µM) + BAP (4.5 µM)	
		Differentiated cells type	% cells	VC (mg/L)	% cells	VC (mg/L)
Photoperiod (hd^{-1})	0	TPWC	23	0	17	0
		TSTPWC	72		79	
		SWCST	5		4	
	8	TPWC	25	0	21	1
		TSTPWC	70		74	
		SWCST	5		5	
	16	TPWC	31	0	25	27
		TSTPWC	64		59	
		SWCST	5		16	
	24	TPWC	38	0	11	38
		TSTPWC	56		61	
		SWCST	6		28	

Analysis at day 25
TPWC: Thin primary-walled cells, TSTPWC: Thin slightly thickened primary-walled cells, SWCST: Secondary-walled cells with spiral thickening

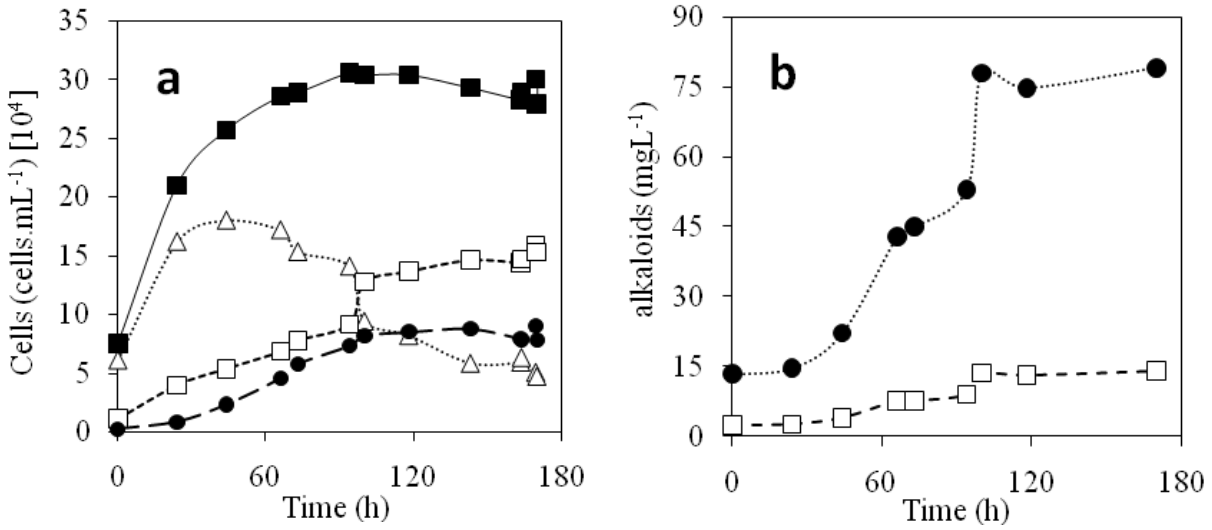

Fig. 3. Kinetic profiles during bioreactor culture producing SWCST cells, VC and VB (first bioreactor culture). (a) Dynamics of total cell concentration (■) and TPWC (△), TSTPWC (□) and SWCST (●) concentrations. (b) Production of vincristine (□) and vinblastine (●). Lines only for eye guide in trend detection.

Unfortunately, since large volumes of sample (25-40 mL) were required for VC and VB analysis, VC and VB concentrations in flasks were only analyzed at the end of the cultures. Consequently, two bioreactor cultures were used to analyze the dynamics of cell differentiation and its impact on VC and VB titers (Fig. 1 d). The first bioreactor was pH and DO controlled, while in the second bioreactor culture, those parameters were monitored but not controlled.

For the first culture in bioreactor, analysis showed that TPWC mainly supported culture growth while TSTPWC were likely to have limited proliferation capacity (Fig. 3 a). On the other hand, as sclerenchyma cells are programmed to die, particularly in the form of tracheary-like elements, increase of cell concentration was only caused by cell differentiation. Cell differentiation to SWCST status was likely to be slow and constant during culture, though cell differentiation to collenchyma from TPWC status was relatively fast, as demonstrated by the abrupt decrease and increase of TPWC and TSTPWC respectively around 100 h of culture. Analysis of VC and VB titer profiles in relation to differentiated cells revealed no positive relationship between TPWC with VC and VB production (Fig. 3 b). During the first 24 h of culture, TPWC increased two-fold but no increase in VC and VB was detected. This fact could explain why fast-growing suspension cultures of *C. roseus* mainly composed of TPWC are usually reported as non-producing VC and VB processes. Direct comparison of TSTPWC and SWCST profiles with VC and VB profiles revealed a likely correlation of VC and VB production to TSTPWC and SWCST status respectively. This is in agreement to previous results in flasks experiments where VC titers were associated to SWCST.

For the second culture in bioreactor, the increase of cell concentration was also supported by TPWC, though the lag phase was longer than in the first culture (Fig. 4 a). Concentration of TSTPWC and SWCST remained practically constant during the culture, except for TSTPWC at the beginning and end of the culture; titers for VC and VB also remained constant with no important increase (Fig. 4 b). Contrary to flask cultures, both bioreactor cultures overproduced VB instead of VC, probably by different inoculum source and culture nature.

4 Discussion

As far as it can be ascertained, SWCST have not yet been reported in *C. roseus* suspension cultures and this is perhaps the first report of *C. roseus* suspension cultures showing empirical data of cell differentiation within cell aggregates based on cell wall differences. Cell heterogeneity in callus or suspension cultures of *C. roseus* in terms of cell morphology or differentiation has only been described in a limited

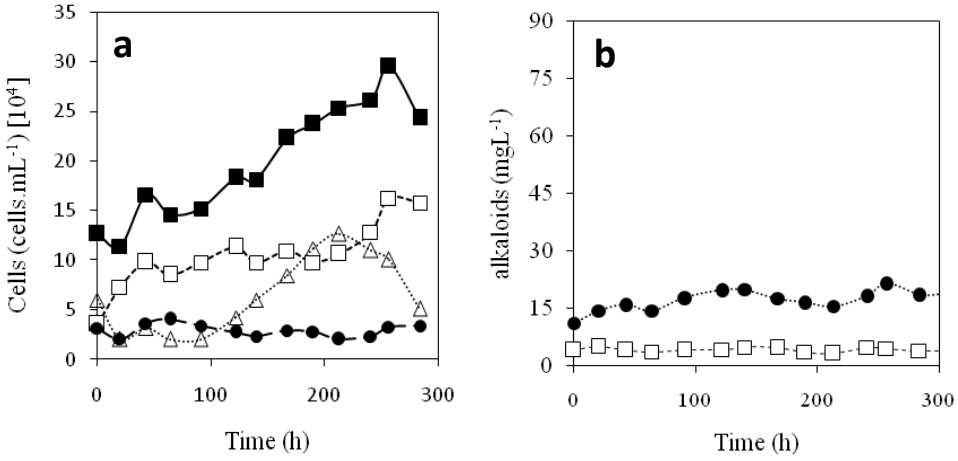

Fig. 4. Kinetic profiles during bioreactor culture not producing either SWCST, VC or VB (second bioreactor culture). (a) Dynamics of total cell concentration (■) and TPWC (△), TSTPWC (□) and SWCST (●) differentiated cell concentrations. (b) Production of vincristine (□) and vinblastine (●). Lines only for eye guide in trend detection.?

way, probably because plant cell growth analysis is usually performed by cell dry weight. In fact, although no consensus has been achieved for the term cell differentiation in suspension cultures, it has been widely stated that cell differentiation in *C. roseus* is favorable for producing molecules of interest (Lindsey & Yeoman, 1983). The size and elongated form of cells have been related to a cell differentiated state and enhanced indole alkaloid production (SukWeon Kim, Jung, Kwak, & Liu, 1994). Other reports have defined cell differentiation in terms of chemical properties of cells. For instance, cell differentiation has been based on different vacuolar pH or on vacuole color due to the presence of alkaloids and other plant metabolites (Knobloch, Bast, & Berlin, 1982; Neumann, Krauss, Hieke, & Gröger, 1983). However, all these reports and the great majority of images provided in publications of *C. roseus* showed relatively homogeneous cell populations of TPWC in terms of cell anatomy (Hall & Yeoman, 1987; SukW. Kim, Song, Jung, Kwak, & Liu, 1994).

Results clearly suggested that differentiation into SWCST is likely related to alkaloid expression and more importantly, to VC and VB synthesis. Analysis of cell differentiation dynamics in bioreactors suggested that the key for VC and VB synthesis is the presence of SWCST and the presence of TSTPWC to a lesser extent in cell aggregates. These results are in agreement with a former study in suspension cultures of *Cinchona ledgeriana*, where differentiated cells as SWCST were located at the periphery of aggregates and contained 90 % of total alkaloid titer (Hoekstra, Harkes, Verpoorte, & Libbenga, 1990). Unfortunately, most studies about cell differentiation in suspension cultures, have mainly focused on woody issues and little is known about alkaloid production.

Several attempts have been made to evaluate the effect of PGR on vincristine and vinblastine production with different results in plants (Pan *et al*., 2010; Srivastava & Srivastava, 2007). Studies using callus cultures have underlined the importance of PGR mixtures (Kalidass *et al*., 2010; Mekky, Al-Sabahi, & Abdel-Kreem, 2018), and so this work focused on evaluating combinations of common auxins and citokines used for suspension cultures and also their effect on different photoperiods. The PGR regimen and light exposure as operating conditions offered a viable way for inducing cell differentiation, particularly into SWCST, and thus VC and VB synthesis (Table 1). For studying wood formation using suspension cultures, plant growth regulators are usually used to promote cell differentiation (Devillard & Walter, 2014; Fukuda, Ito, Sugiyama, & Komamine, 1994; Roberts & Haigler, 1994), though no clear consensus has been achieved. Results also showed that light exposure induced cell differentiation as shown in Table 2. Möller *et al*. (2006) found similar results in callus cultures of *Pinus radiata* where increased light exposure (continuous light or 16 hd^{-1} photoperiod) increased differentiation into SWCST. However, cultures in bioreactors suggested that there may be other important variables affecting cell differentiation such as gas composition and more importantly, the physiological state of inoculated cells. For instance,

in the first bioreactor with DO control (Fig. 3), gas phase composition was partially conditioned by cells and only modified when necessary for maintaining DO at set point, while in the second bioreactor without DO control and with continuous aeration (Fig. 4); gas phase was the same as ambient atmosphere. These differences in gas composition and inoculum used for the bioreactor cultures explained difference performance, though the same PGR regimen was used for both bioreactor cultures. Any cell culture made will be unique due to its physiological state, record of subculture, and inherent heterogeneity (somoclonal variation, aggregates sizes, among others) (Bhatia & Sharma, 2015; Deus-Neumann & Zenk, 1984; Patil & Roberts, 2013). The findings reported here are in line with this. Thus, for optimization of culture conditions seeking cell differentiation and VC and VB synthesis for production processes, the use of experimental design or response surface (Bashir, Asgher, Hussain, Bhatti, 2019; Nisar, Abdullah, Kaleem, Iqtedar, 2019) that enable optimizing the required number of variables in one experiment is recommended.

Results strongly suggested that there is a relationship between VC and VB synthesis capacity and SWCST cells in suspension cultures. This fact implies that cells in suspension cultures globally contained the machinery for VC and VB synthesis provided some degree of differentiation within cell aggregates existed. Indeed, it was recently discovered that *in vitro* cultured cambial meristem cells contained complete sets of enzymes that are responsible for the production of VC and VB from vindoline, though vindoline supplementation was required (Zhang *et al*., 2015). Most enzymes responsible for vindoline synthesis from tabersonine are already elucidated (tabersonine 16-hydroxylase (T16H), O-methyltransferase (OMT), N-methyltransferase (NMT), desacetoxyvindoline-4-hydroxylase(D4H), and deacetylvindoline-4-O-acetyltransferase (DAT))(Yu, Zhu, Wang, & Wen, 2015). However, the enzyme that catalyzes the conversion of 16-methoxytabersonine to 16-methoxy-2,3-dihydro-3-hydroxy- tabersonine is still unknown (Liscombe & O'Connor, 2011). Part of the *in vivo* metabolic pathway for vindoline has been localized in chloroplasts (De Luca & Cutler, 1987) but not usually reported in heterotrophic cultures; however, there have been reports of vindoline-producing cultures under heterotrophic conditions (Naaranlahti *et al*., 1989; Scott *et al*., 1980). Thus, there must be alternative metabolic pathways for vindoline, VC and VB synthesis that could be active either during cell differentiation or in particular differentiated cells.

Vindoline, derived from the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, is usually perceived as the limiting precursor for *in vitro* synthesis of VC and VB. Indeed, it is believed that the last *in vivo* steps for vindoline synthesis occur within idioblast and laticifer cells in aereal tissue, though the principal steps of the MEP pathway for producing tryptamine and secologanin occur in other differentiated epidermal cells, requiring translocation of intermediates for vindoline synthesis (St-Pierre *et al*., 1999). More recently, *in vivo* expression of enzymes of the MEP pathway that were thought to be restricted to aerial tissue were also detected in phloem cells (Burlat, Oudin, Courtois, Rideau, & St-Pierre, 2004). This has implied that *in vivo* expression of a particular enzyme could be undertaken by several types of differentiated cells in different tissues. This fact is likely the case for the suspension cultures that produced VC and VB. The *in vitro* micro-environment of cell aggregates containing TPWC could have mimicked the *in vivo* dynamics of translocation between phloem-aerial tissue to some extent, leading to VC and VB production from vindoline in TPWC (Zhang *et al*., 2015). These phenomena thus require more attention for further research, particularly the identification of the degree of cell differentiation, by using biochemical markers preferably in synchronous cultures for a deeper understanding of cell differentiation phenomena. Moreover, the relationship of the progression of cell differentiation with the expression of key enzymes for vindoline, VC and VB synthesis, as well as the effect of the micro-environment within cell aggregates of differentiated cells, should be addressed.

Conclusions

The production of anticancer molecules (VC and VB) by *C. roseus* cells was investigated in suspension cultures, taking into account for the first time the cell cyto-differentiation analysis of cell aggregates. The effects of various operating conditions on culture performances, including addition of PGR and light exposure, were evaluated. In particular, these culture conditions have been shown to affect cell differentiation. Moreover, this cell differentiation into SWCST, was observed to improve the production of alkaloids such as VC and VB. These results provide important insights into the design of

enhanced production processes. They demonstrated the utility of taking into account cell differentiation for the establishment of advanced processes of cells suspension cultures. Despite these promising results, further studies are still required to better understand the metabolic process of cell differentiation in *C. roseus* suspension cultures.

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