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DOI: 10.2225/vol15-issue4-fulltext-7 *SHORT COMMUNICATION*

Production of phenolic metabolites by *Deschampsia antarctica* shoots using UV-B treatments during cultivation in a photobioreactor

Álvaro Sequeida[1] · Eduardo Tapia[1] · Marcelo Ortega[2] · Pablo Zamora[3] · Álvaro Castro[2] · Christian Montes[4] · Gustavo E. Zúñiga[5] · Humberto Prieto[4] ✉

1 Universidad Técnica Federico Santa María-Pontificia Universidad Católica de Valparaíso, Programa Doctorado en Biotecnología, Valparaíso, Chile

2 Universidad de Santiago de Chile, Programa Doctorado en Biotecnología, Santiago, Chile

3 University of California-Davis, Plant Sciences Department, USA

4 Instituto de Investigaciones Agropecuarias, Centro Regional de Investigación La Platina, Santiago, Chile

5 Universidad de Santiago de Chile, Facultad de Química y Biología, Departamento de Biología, Santiago, Chile

✉ Corresponding author: hprieto@inia.cl
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Abstract *Deschampsia antarctica* (DA), the only species in the Gramineae family endemic to the Antarctic territory, is characterized by a combination of high levels of free endogenous phenylpropanoid compounds under normal *in situ* and *in vitro* growth conditions. In this article, we describe the design and use of a specific temporary immersion photobioreactor to produce both increased DA biomass and secondary metabolite accumulation by UV-B elicitation during cultivation. Three min-long immersions in an induction medium applied every 4 hrs at 14ºC ± 1 and 20/4 hrs light/darkness photoperiod increased DA biomass production over previous *in vitro* reports. Biomass duplication was obtained at day 10.7 of culturing, and maximum total phenolics and antioxidant activity were observed after 14 day of culturing. The addition of UV-B radiation pulses for 0.5 hrs at 6 hrs intervals increased total phenolics and antioxidant activity more than 3- and 1.5- fold, respectively, compared to controls with no UV-B. Significant accumulation of scopoletin, chlorogenic acid, gallic acid and rutin was found in these plantlets. This is the first bioreactor designed to optimize biomass and phenylpropanoid production in DA.

Keywords: *Deschampsia antarctica*, phenolic compounds, temporary immersion bioreactor, UV-B elicitation

INTRODUCTION

In Antarctica, environmental factors such as low temperatures (0 to 6ºC during the summer and 0 to -6ºC during the winter, Alden and Hermann, 1971), limited water availability (Robinson et al. 2003), high salinity and high levels of UV-B radiation (Alberdi et al. 2002) are limiting conditions for most plants. However, these conditions have not affected *D. antarctica* (DA), one of the two flowering plants native to the continent. Many studies have documented the deleterious effects of UV-B on plants, including reduced growth and photosynthesis (Germ et al. 2005; Ruhland et al. 2005). UV-B also places oxidative stress on aerobic cells (Yang et al. 2005; Yannarelli et al. 2006), which leads to the generation of several reactive oxygen species (superoxide anion, hydrogen peroxide and hydroxyl radical), each of which targets different macromolecules (Apel and Hirt, 2004).

One of the most interesting aspects of DA is its ability to generate high levels of compounds derived from the phenylpropanoid pathway, *i.e.* phenolic acids and flavonoids (Ruhland et al. 2005). These

compounds in turn generate antioxidant activity and protective action against UV-B through a selective sunscreen protection that affects targets in the mesophyll. UV-B increases the concentration of flavonoids in DA (Day et al. 2001; Van de Staaij et al. 2002; Ruhland et al. 2005) such as orientin, luteonin and isoswertiajaponin 2''-O-beta-arabinopyranoside (Webby and Markham, 1994; Rozema et al. 2002). Flavonoids act as photoreceptors, anti-microbial, feeding deterrents, metal chelators and antioxidants, protecting plants against factors that induce the oxidative stress and damage caused by free radicals (Kliebenstein, 2004; Ververidis et al. 2007).

Experts in the field of metabolic engineering have shown a great deal of interest in the production of antioxidant compounds (Ververidis et al. 2007), and DA is a very attractive natural source of these compounds. However, limited access to Antarctica and the difficulties that this territory presents for the planning of basic or applied research programs stand as obstacles to the plant's use in biotechnology.

Adequate culture systems offer important operational plasticity, expanding experimental scopes and offering culturing changes that can be measured in real time. Several reports support the use of temporary immersion bioreactors (TIBs) as high efficiency tools for optimizing the production of plant materials (Escalona et al. 1999; McAlister et al. 2005; Etienne et al. 2006). However, very few researchers describe these systems as tools for optimizing secondary metabolite production (Quiala et al. 2006; Arencibia et al. 2008).

In this study, researchers designed and used a TIB for both DA biomass propagation and UV-B light elicitation in order to allow for *in vivo* synthesis and the accumulation of phenylpropanoid-derived compounds.

MATERIALS AND METHODS

Plantlets

DA plants from Robert Island were prepared, transported to the laboratory, stored and propagated as described by Zamora et al. (2010). In this solid-state (S-St) procedure, shoots are stored and propagated using a micro-propagation medium every 21 day. Plantlets from S-St were used as experimental inoculums for TIB experimentation. Inoculums were leaf and root cuts of 1 and 0.5 cm in length, respectively. Between 10 and 15 explants (1.2 g fresh weight) were used as inoculums.

TIB design and construction

The TIB was made from two Pyrex glass vessels connected by stainless steel joints and pipes; an upper vessel (UPV; Figure 1a) containing shoots in culture with a 1 cm water jacket to keep the culturing temperature constant and a lower vessel (LOV; Figure 1b) containing culture media. The system was designed exclusively for DA propagation at bench scale using MS-Visio Software 2010 (Microsoft). The UPV consisted of a cylindrical section and a truncated cone section. The cylindrical section was defined by:

$$V_c = \pi H_i \left(\frac{D}{2}\right)^2$$

[**Equation 1**]

In which H_i is the maximum height reached by DA shoots kept under the S-St system before a new round of propagation (8 cm in average) and D is the diameter of the section.

The volume added to the upper container by the truncated cone was defined by:

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$$V_t = \left(\frac{\pi \cdot h}{3}\right)\left(R^2 + r^2 + R \cdot r\right)$$

[Equation 2]

In which R and r are the radius of the upper and lower sections of the cone, respectively. The total volume of UPV was obtained by adding the two volumes.

The LOV consisted of a cylindrical section and a truncated cone section. However, the volume of LOV only included the cylindrical section of the vessel defined using Equation 1 because the truncated section was used as an over-pressure cavity in order to allow the medium to flow towards the UPV.

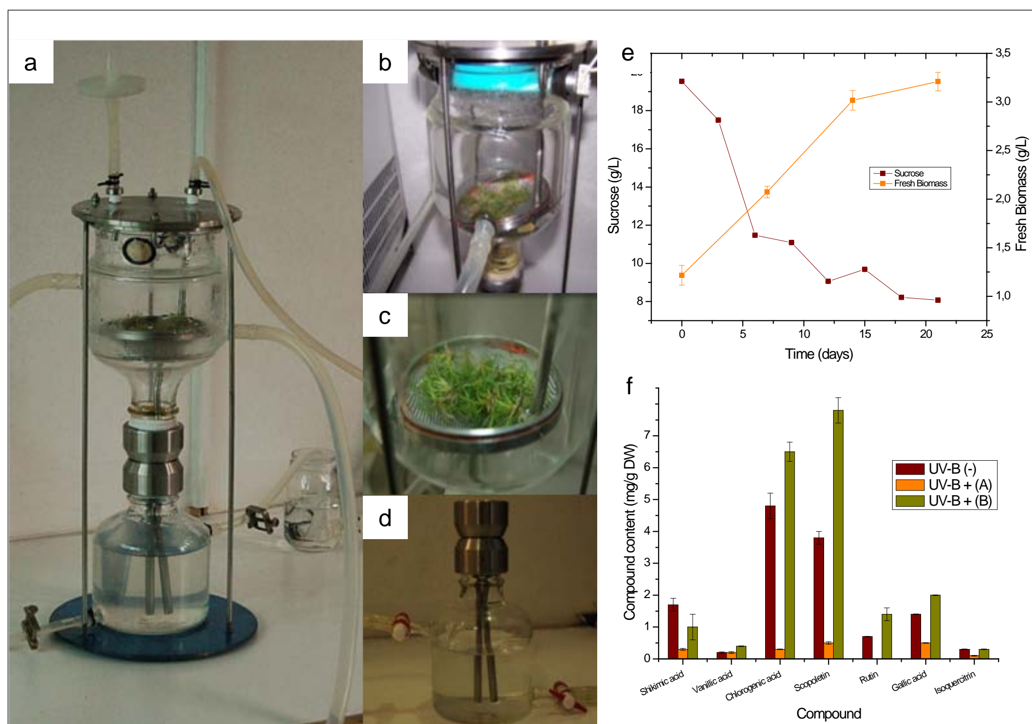

Fig. 1 Temporary immersion bioreactor (TIB) for *D. antarctica* (DA) culturing and phenolic metabolites production by UV-B elicitation. A general overview of the TIB system is shown (a) with upper (b) and lower (d) vessels separated by the grid for DA shoots (c). Biomass production and sugar consumption was measured (e) in order to establish kinetic parameters under control conditions (*i.e.* a 20/4 hrs photoperiod using white light). UV-B elicitation by a lamp located at the upper vessel (b) of TIB was assayed in order to evaluate biomass production, the generation of total phenolic compounds and DPPH radical scavenging capacities of the system (results in Table 1), allowing for the design of culturing procedures for best phenolic compound induction. The presence of known phenylpropanoid-derived compounds by mass spectrometry (f) was compared for white light (20/4 hrs) photoperiod [UV-B (-)], 4 hrs per day (49.6 kJ m^{-2}) UV-B pulses [UV-B + (A)], and 0.5 hrs every 6 hrs [UV-B + (B)] (6.2 kJ m^{-2}) UV-B pulses. For time-course experiments, samples were collected at 0, 7, 14 and 21 day after inoculation (means ± SD). For UV-B inductions, samples were collected 14 day after inoculation in TIB, and treatments were applied on pre-growth biomasses as described in Materials and Methods. gFW: g of fresh weight. Data are shown as means ± SD.

For UPV, the operative volumetric value was 1 L using the length of the UV-B light lamp (13.4 cm, Type G4T5E, Sankyo Denki Co, Ltd., Tokyo, Japan) as the diameter of the vessel. The height of the bioreactor was 60% overestimated due to the addition of inner luminescence and in order to avoid the immersion of the light lamp in culture medium and provide adequate headspace for gas exchange. Compressed air was pumped by a Vento 1000 compressor (Indura S.A. Inc., Santiago, Chile), allowing

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pneumatic culture media to transfer from LOV to UPV. Once interrupted, the culture media flushed back. All of this was controlled by a solenoid valve. The pump tubing was silicon Masterflex L/S LS17 and LS 18 (Cole-Palmer, IL, USA), and the air filters were Millipore FG 0.2 mm (Millipore, Billerica, MA, USA).

TIB operation

The temperature in the water jacket in the UPV was maintained at 14ºC with a Thermo-Circulator Labtech™ circulator (Alexandra Point, Singapore). The shoots were placed in a stainless steel grid (Figure 1c). Culture media were contained in the LOV. The valves allowed for culture media sampling and sucrose consumption monitoring. Immersion duration and frequencies were controlled by a 6-station Orbit® WaterMaster® temporizer (North Salt Lake, Utah, USA) which allowed researchers to open and close a ¾ diameter zero differential pressure solenoid Burket™ valve (Irvine, California, USA).

Culture establishment and UV-B light induction

Initial conditions for DA shoot culturing were set with a frequency of 6 three-min immersions per day. Micropropagation culture media were based on the method described by Zamora et al. (2010) modified for liquid culture supplemented with 0.2 mg/L kinetin and 0.3 mg/L benzylamino purine, henceforth the immersion medium (IM). All procedures were carried out at 14ºC ± 1 and under a 20/4 (day/night) light regime. For UV-B elicitation experiments, the lamp used emitted 0.34 mW cm^{-2} UV-B radiation on the stainless steel grid as experimentally determined by a VLX-3W CE radiometer with microprocessor (Vilber Lourmat, France). UV-B treatments were a pulse of 4 hrs per day at 49.6 kJ m^{-2} imitating peak situations of daily UV-B between noon and 3 pm during Antarctica's springtime (George et al. 2002) (A) or 0.5 hrs UV-B pulses at 6 hrs intervals per day at 6.2 kJ m^{-2} (B).

Assays

DA shoots were picked at 7, 14 and 21 days post-inoculation (dpi) in the UPV. In addition, 5 mL of IM was sampled every 2 days. Plantlets were transferred to filter paper in order to eliminate excess liquid for 45 min at room temperature and weighed. The sucrose content of IM samples was determined using acid hydrolysis methodology (Marques and Farah, 2009). For experiments in which UV-B led to the induction and identification of specific phenolic compounds, cultures were first subjected to a control white light photoperiod (20/4 hrs) in order to obtain 7-day growth biomasses. Additional 7 days of culturing under the specific UV-B treatments was then carried out.

Total phenolics extraction

Phenolics were extracted using a modified methodology based on Lin and Harnly (2007).

Total phenolics content

The total phenolics compound content was determined using a modified Folin-Ciocalteu colorimetric method (Asami et al. 2003).

Antioxidant activity

Free radical scavenging activity of methanolic extracts was measured using the stable purple chromogen 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Sigma-Aldrich, St. Louis, MO, USA) in the photometric assay adapted from Grzegorczyk et al. (2007). Free radical scavenging capacity was calculated and expressed using the equation:

% antioxidant activity = [(Absorbance of blank-Absorbance of sample)/Absorbance of blank] * 100

Volumetric productivity (Qx)

DA volumetric productivity was calculated by determining fresh weight in time-course analyses. Samples were picked at days 0, 7, 14 and 21 of cultivation. Three separate culturing batches were started using 1.2 g of cultured shoots.

Phenolics volumetric productivity (Qp)

Phenolics volumetric productivity was calculated as indicated by Asami et al. (2003). All plant material was sampled from the bioreactor at days 0, 7, 14 and 21 dpi. Three separate culturing batches were started using 1.2 g of cultured plantlets.

Analysis of phenolic compounds by LC/MS

A 6490 Triple Quadrupole LC/MS equipped with an ESI source (Agilent Technologies, Palo Alto, CA, USA) was used. All determinations were performed under negative ionization mode with a capillary voltage of 3000 V. Nitrogen was used as the nebulizer gas (35 psig) and drying gas at 10 L/min at a temperature of 300ºC. The PMT, fragmenter and skimmer were set at 850, 100 and 60 V, respectively. A full scan mass spectrum was acquired from m/z 100 to 1300. Data acquisition and processing were carried out using MassHunter MS Optimizer software (Agilent Technologies, Palo Alto, CA, USA). Separation was achieved on a C18 column; 150 x 4.6 mm I.D.; 5 µm particle size (Agilent Technologies, Palo Alto, CA, USA). The mobile phase consisted of water with 0.1% formic acid (a) and acetonitrile with 0.1% formic acid (b) at a flow rate of 0.5 mL/min. The linear elution gradient was from 80% (a) to 60% (a) in 20 min. Each run was followed by a 5 min wash with 100% (b) and an equilibration period of 11 min with 80% (a) /20% (b). The total run time for analysis was 20 min. Ten microliters of the sample were injected and peaks were to be assigned based on the mass of the standard compounds (Sigma).

Statistical analyses

All data were subjected to variance analyses. Means were separated using an LSD test at 5% significance using Statgraphics Centurion XV (Manugistics Inc., Rockville, MD, U.S.A.). Analysis of the specific growth rate (µ) in Table 1 was carried out using Tukey's test from the same software package. The results are shown as mean ± standard deviation (SD).

Table 1. Effect of UV-B light on *D. antarctica* TIB culturing. Determinations were made based on *D. antarctica* (DA) plantlets cultured for 7 and 14 days under 20/4 white light photo-period [UV-B (-)], a pulse of 4 hrs per day [UV-B + (A)] (49.6 kJ m-2), and a pulse of 0.5 hrs of UV-B every 6 hrs [UV-B + (B)] (at 6.2 kJ m-2). Samples were collected at 7 and 14 days post-inoculation (dpi) (means ± SD). gFW: g of fresh weight; GAE: gallic acid equivalent; Q_x: biomass volumetric productivity; Q_p: phenolic compounds volumetric productivity; µ: DA specific growth rate. Tukey's test was applied for analysis of µ.

Determination	UV-B(-)		UV-B(A)		UV-B+(B)	
	7 dpi	14 dpi	7 dpi	14 dpi	7 dpi	14 dpi
Qx(gFW·d^{-1}·L^{-1})	-	0.22 ± 0.001	-	0.17 ± 0.01	-	0.19 ± 0.01
Rad. Sc on DPPH (%)	17.75 ± 0.75	23.37 ± 1.3	16.01 ± 0.75	18.60 ± 3.7	36.17 ± 0.7	38.61 ± 1.40
Total Phenolics [(GAE/mL)gFw]	4.32 ± 0.21	5.08 ± 0.15	3.28 ± 0.32	3.41 ± 0.47	13.93 ± 0.15	14.61 ± 0.43
Qx(mg·d^{-1}·L^{-1})	-	0.34 ± 0.02	-	0.22 ± 0.03	-	0.97 ± 0.02
µ (d^{-1})	0.065a		0.056a		0.064a	

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RESULTS AND DISCUSSION

Previous micropropagation procedures for DA consisted of 21 days long sub-culturing cycles in IM (Zamora et al. 2010). Neither biomass gain nor active budding is experimentally obtained beyond this period. The use of an *ad-hoc* TIB (Figure 1a) allowed for the characterization of biomass production during this culturing (Figure 1e). Starting batching conditions in TIB led to a peak in biomass production of 3.0 ± 0.1 g/L after 14 days of cultivation with no significant biomass production beyond this stage. The biomass duplication rate was obtained from these data at around 10 days post inoculation (dpi). A high rate of sucrose consumption up to 5 dpi (Figure 1e) was observed. Although the synthesis of phenolic compounds observed between 7 and 21 dpi showed no statistical differences during that period (not shown), the highest value (5.1 ± 0.1 mg of GAE per mL/g FW) was determined at day 14. Interestingly, a peak of 23% in radical scavenging on DPPH was clearly established at 14 dpi as well (not shown), while antioxidant activity was dramatically reduced at the end of batching (21 dpi, 14.3 ± 0.01%). These data suggested a stationary phase of DA cultivation with maximal biomass production from 14 day of cultivation. The reduction in sucrose consumption rates observed after 5 dpi fits perfectly with the assumption that secondary metabolism is triggered (Collin, 2001), *i.e.* carbon skeletons and primary metabolites generated during the first part of the time-course (days 1-5) are linked to the implementation of metabolic processes unrelated to biomass duplication and closer to secondary metabolism pathways (days 6-14).

Analysis of induced compounds under simulated UV-B stress conditions was performed by comparing DA batches obtained after control [UV-B (-)] and two [UV-B (+)] situations in which the maritime Antarctic conditions during the plant's growing season (*i.e.* up to 137 kJ m^{-2}, (A)) were compared to a milder radiation presence (6.2 kJ m^{-2}, (B)). The specific growth rate (μ) for the control situation was 0.065 * d^{-1} (Table 1), which led to a Q_x of 0.22 $gFW{*}d^{-1}{*}L^{-1}$ at 14 dpi. The attenuated UV-B treatment (B) markedly reduced the total biomass yield to 0.19 ($gFW{*}d^{-1}{*}L^{-1}$) in the same term despite a similar μ of 0.064 d^{-1} (Table 1) that duplicated the biomass at 10.8 dpi. The intensive UV-B treatment (A) did not have a marked effect on biomass production prior to 14 dpi, and biomass did not begin to be duplicated until 12.4 dpi (Q_x = 0.17 $gFW{*}d^{-1}{*}L^{-1}$). As a complement to these kinetic determinations, Q_p of phenolic compounds was defined using TIB culturing in the presence of UV-B. Attenuated treatment led to important increases in Q_p value in comparison to the control and extreme UV-B conditions. The content of phenolic compounds and radical scavenging activities in methanolic extracts confirmed that control cultures had increased amounts of total phenolic compounds and DPPH scavenging activity at 14 dpi compared to similar batching at 7 dpi (Table 1). However, (A) had a profoundly detrimental effect on both determinations at both batching times (Table 1). In contrast, the concentration of total phenolics under (B) showed a three-fold increase on average compared to control conditions at both dpi, and antioxidant activity showed a two-fold increase on average compared to the same controls at both dpi.

Table 1 reveals a cultivation step of up to 14 dpi in which biomass production with no visual impact on explants' viability due to (B) is observed. An important synthesis of secondary metabolites is translated into higher Q_p values than those observed in control situations. The use of this system for production of phenylpropanoid-related bioactive compounds was assayed by continuous 14-day long cultures. Pre-growth batches in which a DA biomass was grown by 7 day of culturing were subjected to treatments (A) and (B) during 7 day of additional batching. Mass spectrometry analyses of plantlets extracted from (B) treatments showed significant accumulation of compounds such as scopoletin and, to a lesser extent, chlorogenic acid, gallic acid and rutin. In contrast, and according to the data gathered by applying (A), extremely low production of the evaluated compounds was recorded in all cases.

The molecules identified through the process outlined above are described as antioxidants. These elements are commonly used in the pharmaceutical industry in sunscreens and in the food industry as food enhancers (Wang and Luo, 2007; Bayoumi et al. 2008; Marques and Farah, 2009). It seems that the increase in these compounds may be a metabolic defensive response that is meant to restore the equilibrium between antioxidants and reactive oxygen species due to the oxidative burst caused by exposure to UV-B radiation (Zhang and Björn, 2009; Zamora et al. 2010). Researchers have already established that chlorogenic acid shows a marked tendency to accumulate in plant tissues that are subjected to UV-B radiation (Demkura et al. 2010), shielding the underlying tissue from harmful UV radiation (Jaakola et al. 2004). Furthermore, it seems that there is a direct link between rutin accumulation and elicitation by UV-B radiation in some plant tissue cultures (Zhang and Björn, 2009), which is consistent with the results obtained in this study. Conversely, no accumulation of these compounds was obtained during (A), which supports the idea that this treatment causes important

damage to the explants. To our knowledge, this is the first report describing the use of TIB for elicitation of this type of compound in a tissue culture of DA using UV-B radiation.

In conclusion, a successful automation of *in vitro* culturing for DA has been achieved using a specific TIB. This system allowed for the proliferation and induction of the generated biomass and synthesis of phenolic compounds under elicitation with UV-B. Note that every parameter defined in this article is merely a first approach to the culture and induction of this species under *ex-situ* conditions, which were established in order to explore technical opportunities for basic and applied experimentation. Although the immersion schedule (duration and frequency) and the general culturing conditions generated satisfactory results (handling of DA growth and secondary metabolism), they should be optimized in future studies. Certainly, DA culturing in TIB creates opportunities for producing enough biomass to conduct both basic and applied research. The use of this extremophile in the bio-production of interesting biotechnology compounds, such as chlorogenic acid, now seems plausible.

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