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Production of anthraquinones by *in vitro* culture of plant cells and hairy roots from *Rubia tinctorum*: Model for the teaching of bioprocesses in courses of plant tissue culture

Producción de antraquinonas por cultivo *in vitro* de suspensiones celulares y raíces transformadas de *Rubia tinctorum*: un modelo para la enseñanza en el estudio de bioprocesos por cultivo de tejidos de plantas

M.Perassolo[1,2], V. D. Busto[1,2], J.M. Minoia[1,2], J. Cerezo[1,2], M.E. Smith[1,2], C.A. Martínez[1], C.V. Quevedo[1,2], A.M. Giuletti[2], J. Rodríguez-Talou[1,2*], A.B. Cardillo[1,2]

[1] *Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Departamento de Microbiología, Inmunología, Biotecnología y Genética, Cátedra de Biotecnología, Buenos Aires, Argentina.*
[2] *CONICET-Universidad de Buenos Aires, Instituto de Nanobiotecnología (NANOBIOTEC), Buenos Aires, Argentina.*

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Abstract

Plant *in vitro* cultures are an attractive and feasible alternative to produce secondary metabolites. Plant cultures assure a continuous product supply and standardized production according to Good Manufacturing Practice requirements. These bioprocesses also constitute an excellent tool for training postgraduate students. In fact, students can gain experience in different fields, such as plant biochemistry, molecular biology, plant primary and secondary metabolism, and biochemical engineering. In this work, anthraquinone production in different plant *in vitro* cultures of *Rubia tinctorum* is presented as a straightforward and versatile model to introduce postgraduate students into plant secondary metabolite production. The students were able to analyze biomass and product formation kinetics in batch cultures of cell suspensions and hairy roots, and evaluate the effect of methyljasmonate elicitation, a well-known strategy used to trigger secondary metabolite production in plant *in vitro* cultures. The performance of *in situ* product removal, extensively used in biocatalysis and microbial fermentations, was also evaluated. Students' self-evaluation revealed that laboratory work and subsequent data analysis helped them to reinforce the theoretical concepts acquired during lectures. In summary, an inexpensive, accessible, and robust laboratory work is presented that can be used in postgraduate courses in different plant biology, chemistry, and biochemical engineering programs.

Keywords: laboratory work, hands-on learning, secondary metabolite production, plant cell culture, *in situ* product removal.

Resumen

El cultivo vegetal *in vitro* es una alternativa atractiva y factible para la producción de metabolitos secundarios. Este tipo de cultivos asegura una producción continua en condiciones controladas, bajo Buenas Prácticas de Fabricación. Estos procesos biotecnológicos constituyen una excelente herramienta para la formación de estudiantes de postgrado. Efectivamente, los estudiantes pueden adquirir experiencia en diferentes áreas, tales como bioquímica vegetal, biología molecular, metabolismo primario y secundario vegetal e ingeniería bioquímica. En este trabajo, la producción de antraquinonas en diferentes cultivos vegetales *in vitro* de *Rubia tinctorum* se presenta como un modelo sencillo y versátil para introducir a los estudiantes de postgrado en la producción de metabolitos secundarios vegetales. Los estudiantes pudieron analizar la cinética de formación de biomasa y productos en cultivos en batch de suspensiones y raíces transformadas, evaluando el efecto de la elicitación con metiljasmonato, una estrategia muy conocida utilizada para inducir la producción de metabolitos secundarios en cultivos de células vegetales. Además, se evaluó el efecto de la remoción *in situ* del producto, ampliamente utilizada en biocatálisis y fermentaciones microbianas. El resultado de las autoevaluaciones reveló que el trabajo en el laboratorio y el subsiguiente análisis de resultados favorecieron la consolidación de conceptos teóricos adquiridos durante los seminarios. En resumen, se presenta un trabajo de laboratorio económico, accesible y robusto, aplicable a estudiantes de cursos de postgrado en diferentes programas de biotecnología vegetal, química e ingeniería bioquímica.

Palabras clave: trabajo de laboratorio, aprendizaje práctico, producción de metabolitos secundarios, cultivo de células vegetales, remoción *in situ* de producto.

* *Corresponding author. E-mail*: jrtalou@ffyb.uba.ar
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1 Introduction

Plants are a source of numerous natural compounds used in the production of fine chemical and pharmaceutical products. In general, these compounds are extracted from plants grown in the field, a practice that implies some disadvantages, such as slow growth and yield variability due to climatic and seasonal factors and presence of pathogens (Lubbe & Verpoorte, 2011). In addition, traditional collection methods may result in a non-sustainable management of these natural resources, a fact that puts many plant species at risk of extinction (Ramirez-Estrada *et al*., 2016; López-Ramírez *et al*., 2021). On the other hand, chemical synthesis of these products is not often feasible due to their complex structures, which would require multiple synthesis and purification steps (Halder *et al*., 2019). In addition to this, reaction products are usually a mixture of isomers and epimers that compromise the biological activity of the desired product, resulting in less efficient, less environmentally friendly and more expensive processes (Mora-Pale *et al*., 2013). For these reasons, the *in vitro* culture of plant cells, tissues and organs constitutes an attractive and feasible alternative for the production of secondary metabolites. Bioprocesses are carried out under controlled conditions (including good manufacturing practices), without risk for the environment, according to green chemistry procedures (Marchev *et al*., 2020). In this sense, secondary metabolites can be obtained using the biosynthetic capacity of the plant cells or tissues (de novo synthesis). Moreover, the risk of bioaccumulation of toxics in plants grown in contaminated soils is avoided. Although *in vitro* culture has allowed obtaining many secondary metabolites successfully, only a few have given rise to industrial processes. The main obstacles to this are the low levels of production achieved and the difficulty in performing a proper scaling up of the process (Steingroewer *et al*., 2013; Espinosa-Leal *et al*., 2018). At the beginning of the development of this technology, cultures were undifferentiated cell suspensions generated from the treatment with plant growth regulators (PGRs) that were able to grow either as single cells or as aggregates of different sizes (Zavala-Ortiz et a., 2021). This type of cultures allowed the scaling-up in bioreactors used for microorganisms in the biotechnology industry. However, the established cell lines may lose the biosynthetic capacity over time due to somaclonal variation, significantly decreasing productivity (Halder *et al*., 2019). Moreover, in many cases the levels of accumulation of compounds are very low, due to the fact that the biosynthesis of secondary metabolites is associated with the degree of cell differentiation (Murthy *et al*., 2014). In this context, the *in vitro* culture of organs such as shoots or roots has emerged as an alternative. Shoot culture can be used when it is desired to obtain metabolites produced in the aerial parts of a plant, such as monoterpenic essential oils from *Mentha piperita*, whereas root culture can be used when it is desired to obtain tropane alkaloids, such as those from *Brugmansia candida* or *Hyoscyamus muticus* (Hilton *et al*., 1995; Dehghan *et al*., 2012).

The *in vitro* culture of adventitious roots has some disadvantages because it requires the addition of the suitable PGRs to the culture medium, tends to be difficult to establish, and has slow growth rates. In contrast, the culture of transformed roots ("hairy roots"), which are a special type of roots resulting of the interaction of plant cells with the bacterium *Agrobacterium rhizogenes*, is characterized by a high growth rate without the need of adding PGRs, absence of geotropism, high degree of branching, and ability to produce secondary metabolites for long periods of time at levels similar to those found in the plant of origin (Gutierrez-Valdes *et al*., 2020). The productivity of *in vitro* cultures of plants can be improved by applying different strategies, including the addition of elicitors and the *in situ* product removal (ISPR) technique.

Elicitors are agents able to induce physiological changes in plant cells and trigger a defense response that usually involves the accumulation of secondary metabolites and the induction of the expression of genes associated with biosynthetic pathways and of genes involved in the defense against pathogens (Vasconsuelo *et al*., 2006). According to their origin, elicitors are usually classified as biotic or abiotic. Biotic elicitors are of biological origin and may be molecular components of a pathogen (oligosaccharides, peptides, proteins, lipid derivatives) or compounds produced by the plants in their defense response (in this case called endogenous elicitors). These elicitors induce secondary metabolite biosynthesis in a way that resembles the effect that pathogens have on the plant. On the other hand, abiotic elicitors include various physical factors (such as radiation, hydrodynamic stress, etc.) and chemical compounds (such as heavy metals) (Ochoa-Villarreal *et al*., 2016; Sánchez-Rangel *et al*., 2014). The

response to an elicitor may be affected by several factors, including the specificity, concentration and time of the treatment and culture conditions (stage of development, composition of the culture medium, etc) (Halder *et al*., 2019). Some of the most widely used elicitors are jasmonic acid and its methyl ester methyl jasmonate (MeJa), both of which occur naturally in plants and are related to the defense response (Ramirez-Estrada *et al*., 2016).

In any biotechnology process, the recovery and purification of the product are key steps, which often represent a high percentage of the total cost of the process. This issue becomes critical considering that secondary metabolites are usually accumulated inside plant cells and only a very small percentage of them are released to the extracellular medium. Moreover, the slower growth of plant cultures compared with microbial ones poses another constraint to the feasibility of the biotechnological process. ISPR is a technique used in bioprocesses that consists in the continuous removal of the product during the growth culture within a second phase that can be liquid (solvents), solid (resins) or gaseous. This technique has several advantages, including the fact that it prevents the degradation of the product, the potential negative feedback in the biosynthesis, and the toxic effects of the product, and allows a quick and easy recovery of the compound (Vidal-Limon *et al*., 2018; Syklowska-Baranek *et al*., 2018).

In the framework of the postgraduate course "Production of compounds of pharmaceutical interest by *in vitro* culture of plant tissues", included in the program of the Master in Biotechnology of the University of Buenos Aires (Buenos Aires, Argentina), we designed a laboratory practical work with the aim that the students develop skills in plant *in vitro* cultures, secondary metabolite production and the uses of different strategies to optimize these bioprocesses. In every course of biotechnology, the laboratory work is essential for the students, in order to develop and improve their skills in the handling and processing of samples, and in the use of laboratory instruments and equipment.

The experimental work and the subsequent analysis of the results obtained strengthen the construction of knowledge, and together with the concepts acquired during the lectures, promote the development of critical thinking (Fry *et al*., 2000). The design of the laboratory work must be inexpensive, feasible to be performed in different laboratories and, as far as possible, environmentally friendly. Given the great heterogeneity of students and their practical skills in the laboratory, it must also be robust. In addition, it should require the use of elements and equipment that pose no risk for the students.

R. tinctorum is a species belonging to the Rubiaceae family that produces different types of secondary metabolites, being AQs the most important ones. Several species containing these compounds have traditionally been used for medicinal purposes (Han *et al*., 2001). The biological activities attributed to AQs include antitumor, anti-inflammatory, antimicrobial and antioxidant, among others (Diaz-Muñoz *et al*., 2018). As a model for the design of this laboratory work, we decided to use cultures of cell suspensions and transformed roots from anthraquinone (AQ)-producing *Rubia tinctorum* plants.

In addition to being used as colorants by the industry, AQs have potential use in the food industry due to their resistance to different physical agents such as light and temperature (Han *et al*., 2001). The core chemical structure of these secondary metabolites is 9,10-AQ (9,10-dioxoanthracene), which can be substituted in various ways, generating a wide variety of structures. On the other hand, AQs can be extracted from the plant tissue easily and can be determined in a simple way by spectrophotometry. AQs are extracted with 80% ethanol, which is also environmentally friendly and safe to handle in the laboratory. On the other hand, the fact that cell and root cultures are colored (yellow to dark orange) according to the accumulation levels of AQs, allows the direct observation of the effects of elicitors and ISPR.

The objectives considered in the design of this practical work are: To help students acquire the tools to estimate parameters such as growth index, specific production, and productivity; to help students learn about the effect of the elicitors as a strategy to increase the production of secondary metabolites in plants and to make students become familiar with the ISPR technique as a strategy to recover secondary metabolites from *in vitro* plant tissue culture. Students will measure biomass growth and AQ content as a consequence of the strategies applied (elicitation with different concentrations of MeJa and ISPR with hexadecane), as an example of how these treatments influence plant behavior for the production of secondary metabolites of interest.

2 Material and methods

2.1 Chemical reagents

WPM and Gamborg´s B5 medium were purchased from Phytotech and the PGRs used for supplementation of media obtained from Sigma. MeJa was purchased from Aldrich, ethanol from Sintorgan, hexadecane (HXD) from Sigma-Aldrich, Alizarine from MP Biochemicals and sucrose from Biopack.

2.2 Experimental protocol

Due to the long times required to establish and culture cell suspensions and hairy roots, the professors inoculated the cultures before the initiation of the course. The cultures are prepared in such a way that, during the first week of the course, the cultures of cell suspensions and roots are 4 days and 21 days old respectively. Both types of cultures were elicited with the addition of MeJa. The elicitation period for the cell suspensions was 3 days, whereas for the transformed roots was 4 days. In the case of hairy roots, ISPR with HXD was performed in both elicited and control roots. The experiments were planned in such a way that cell suspensions and hairy roots were harvested the same day (third day of the second week of the course). After harvesting the samples, the students determined their fresh weights (FW), froze them in liquid nitrogen, and stored them at -70°C for further processing. AQs were extracted from root and cell tissues with 80% ethanol at 80°C and further quantified by spectrophotometry at 434 nm.

2.3 Plant cell cultures

Rubia tinctorum L. suspension cultures were a kind gift of Dr. Rob Verpoorte (Leiden University, The Netherlands). Cells were cultured in Gamborg's B5 medium, supplemented with 20 g/L sucrose, 2 mg/L 2,4-dichlorophenoxyacetic acid, 0.5 mg/L, 1-naphthaleneacetic acid, 0.5 mg/L indole acetic acid, and 0.2 mg/L kinetin, pH: 5.75-5.80. Suspension cultures were grown in 60 mL of medium contained in 250 mL Erlenmeyer flasks at 25 ± 2°C on a gyratory shaker at 100 rpm with a 16-h photoperiod using cool white fluorescent lamps at a light intensity of 1.8 W/m^2. Subculturing was carried out every 7-10 days, using a three-fold dilution of cells. All the experiments were carried out with cell suspensions cultured as mentioned. Hairy root cultures of *R. tinctorum* were established and cultured according to Perassolo *et al*. (2017) and were subcultured every 30 days in the same media and grown in 250 mL Erlenmeyer flasks at 25 ± 2°C on a gyratory shaker at 100 rpm with a 16-h photoperiod using cool white fluorescent lamps at light intensity of approximately, 1.8 W/m^2.

2.4 Analytical techniques

For fresh weight (FW) determination, plant cells were harvested, washed once with distilled water and filtrated under gentle vacuum for 2 minutes. For fresh weight determination, plant cells and roots were harvested according to Perassolo *et al*. (2017). Final biomass was expressed as gFW/L, considering the FW determination achieved and the volume of medium in each Erlenmeyer (25 mL).

Volumetric productivity (Q_P); Q_P = P/t, were calculated for both processes; were P: product concentration (μmol AQ/L culture medium), and t: overall time of the process (in days). Q_P was expressed as μmol/L.d.

Intracellular (IC) AQs content was determined spectrophotometrically at 434 nm, according to Perassolo *et al*. (2017). Briefly, plant samples were grinded to a fine powder with liquid nitrogen, using a mortar and a pestle. Approximately 200 mg of fresh cell biomass or 50 mg of fresh root biomass were extracted twice with 1.3 mL 80% ethanol for 30 min at 80°C. The extracted fractions were collected and measured spectrophotometrically. For extracellular (EC) AQ content, a sample from culture media was centrifuged at 13000 rpm for 5 min prior to spectrophotometric determination at 434 nm. AQ content was calculated using the molar extinction coefficient of alizarin (5.5 mM^{-1} cm^{-1}), the most abundant AQ in *R. tinctorum*. Results were expressed as Specific AQ content (μmol/gFW) and Volumetric AQ content (μmol/L), considering mass of tissue samples (g) and extraction volume (2,6 ml) or volume of culture medium (1 ml), and final biomass achieved (expressed in gFW/L).

The concentration of AQs in hexadecane (HXD) was determined by measuring the absorbance of samples at 434 nm. A calibration curve of alizarin in HXD (10-120 μM) was used as a reference (R^2 = 0.953).

2.5 Elicitation with Methyl Jasmonate (MeJa) and ISPR

R. tinctorum cell suspensions were inoculated with

approximately 2.5 g of FW into 25 mL of fresh medium, contained in 100 mL Erlenmeyer flasks. Suspension cultures were incubated for 4 days in the conditions mentioned above, and after this time period, cells were treated with different concentrations (100, 200 and 500 μM) of MeJa. For elicitation and ISPR experiments, *R. tinctorum* hairy roots were inoculated into 100 mL Erlenmeyer flasks (0.25 gFW per Erlenmeyer) containing 25 mL of WPM fresh medium. After 21 days of culture, MeJa was added to half of the flasks to a final concentration of 100 μM and ethanol (diluent of MeJa) was also added to the other set of flasks. Control experiments (without any additive) were also carried out. For ISPR experiments, sterile filtrated HXD was added at final concentration of 8% (2 mL per 25 mL of culture medium) three days after MeJa addition. Cell suspension cultures (four days old) were elicited with MeJa at concentrations of 100, 200 and 500 μM. For all the treatments, cultures were sampled at 3 days post elicitation (dpe) for cell suspensions and 4 dpe for hairy roots. For each time point and each condition, three complete Erlenmeyer flasks were harvested.

2.6 *Statistical analysis*

Significance of treatment effects was determined by using Student's test and two-way variance analysis (two-way ANOVA). Variations between treatments means were analyzed by using Tukey's test (p = 0.05). The software used for these analyses was InfoStat 2010 Version. All experimental data were expressed as mean ± SD of three independent replications.

3 Results and discussion

3.1 *Elicitation of Plant suspension cultures*

The effects of different concentration of MeJa were evaluated in cell suspension cultures. As shown in Figure 1B, MeJa significantly ($p < 0.05$) increased specific IC AQs content at all concentrations tested (100, 200 and 500 μM). AQs content was 92% (1.8 μmol/gFW), 127% (2.15 μmol/gFW), and 117% (2.08 μmol/gFW) higher than control cultures (0.95 μmol/gFW) at 100, 200 and 500 μM MeJa, respectively. The presence of the elicitor also decreased biomass growth, especially at 200 and 500 μM MeJa (18 and 38% less biomass than control cultures, respectively). These results correlated with

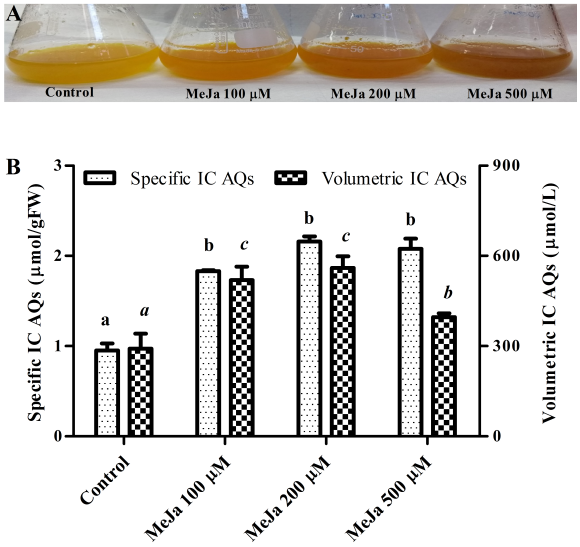

Figure 1. Effects of MeJa elicitation in cell suspension cultures of *R. tinctorum*. (A) Coloration change of cells under control and the different MeJa treatments. (B) IC AQs content of cell suspensions after elicitation with MeJa. Results are depicted as specific production (μmol per gram of fresh weight) and volumetric production (μmol per liter of culture medium). Results are shown as mean ± SD of three independent samples. For each variable, different letters represent significant differences among treatments using Student´s Test ($p<0.05$).

a decrease in cell viability evaluated by Evans Blue uptake (data not shown). The volumetric IC AQs content showed an optimum when cell suspensions were elicited with 100 or 200 μM of MeJa. AQs reached a value of 519.3 μmol/L (at 100 μM MeJa) and 560.0 μmol/L (at 200 μM MeJa) which represented significant ($p < 0.05$) increments of 78% and 92%, respectively. As expected, the color intensity of cell suspensions increased with the increase in elicitor concentration (Figure 1A). These results show that there is an optimum elicitor concentration that maximizes secondary metabolite production, and that a further increase has a negative impact. On the other hand, EC AQs accumulation was not detected in culture medium, thus demonstrating that MeJa had no effect in the release of AQs.

3.2 *Elicitation and ISPR in hairy root cultures*

In order to evaluate the effects of HXD (8% v/v) and MeJa (100 μM) on AQ production in *R. tinctorum*

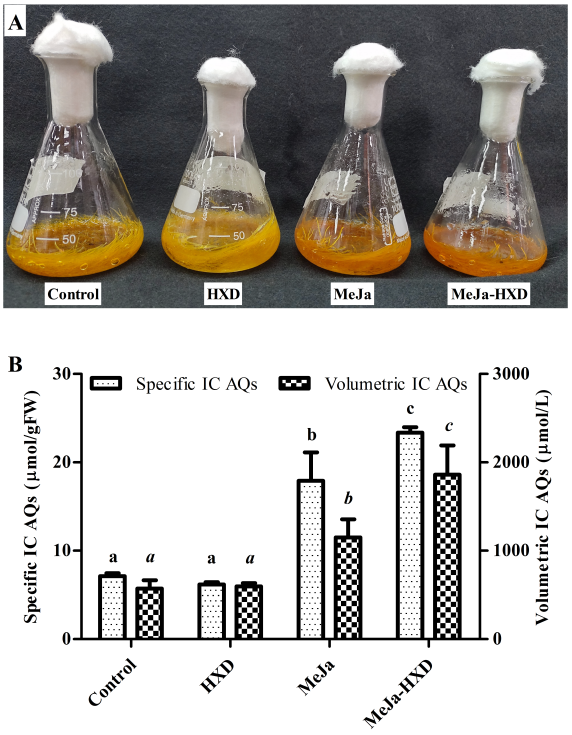

Figure 2. Effect of MeJa elicitation and HXD addition on IC AQ production in hairy root cultures of *R. tinctorum*. (A) Tissue coloration change in hairy roots of *R. tinctorum* after control, MeJa and ISPR treatments. (B) IC AQs content of hairy roots after the treatments (control, HXD, MeJa and MeJa-HXD). Results are depicted as specific production (μmol per gram of fresh weight) and volumetric production (μmol per liter of culture medium). Results are shown as mean ± SD of three independent samples. For each variable, different letters represent significant differences among treatments using Tukey´s Test ($p<0.05$).

Table 1. Full factorial design of the experiment for the combination of 2 factors (MeJa and HXD) in two levels: 0 and 100 μM for MeJa and 0 and 8 % for HXD.

Experiment	Concentration	
	HXD (%)	MeJa (μM)
1	0	0
2	8	100
3	0	0
4	8	100

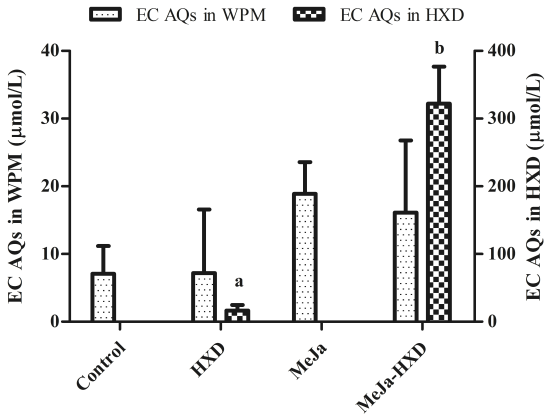

Figure 3. EC AQs content in hairy root cultures of *R. tinctorum* in control, MeJa and ISPR treatments. Results are depicted as volumetric production (μmol per liter), in the medium fraction (EC AQs in WPM), and in the HXD fraction (EC AQs in HXD). Results are shown as mean ± SD of three independent samples. For each variable, different letters represent significant differences among treatments Tukey´s Test ($p<0.05$).

transformed root cultures, we performed a 2^2 full factorial design experiment (Table 1). Figure 2A shows tissue coloration change and AQs accumulation after elicitation and ISPR. Biomass growth was not affected by the addition of MeJa and HXD (data not shown). This solvent was chosen because of its octanol:water (log P) partition coefficient. Solvents with a log P greater than 4 have greater biocompatibility with plant cells (Malik *et al.*, 2013).

MeJa-treated hairy roots reached specific IC AQ content of 16.8 μmol/gFW, which represented a 2.4-fold increase compared with control roots (7.11 μmol/gFW, Figure 2B). The combination of MeJa and HXD addition showed a synergistic effect that resulted in an AQs content of 23.1 μmol/gFW, a 3.3-fold increase compared with control cultures (Figure 2B). HXD-treated cultures did not show a significant difference in IC AQ accumulation compared with the control treatment. The volumetric production (μmol/L) in the combined treatment of MeJa/HXD showed the highest AQs accumulation (1741 μmol/L) compared with those of MeJa (1139 μmol/L), HXD (584 μmol/L), and control cultures (574 μmol/L) (Figure 2B).

Addition of HXD had a deep impact in EC AQ accumulation, especially when it was combined with MeJa elicitation (Figure 3).

Table 2. Two-Way ANOVA for MeJa elicitation and HXD addition on IC AQs specific production (μmol/gFW) in hairy root cultures of *R. tinctorum*.

Variable	*SS*	*DF*	*MS*	*F value*	*p-value(*)*
Model	599.38	3	199,79	84.45	<0,0001
MeJa	536.23	1	536.23	226.95	<0,0001
HXD	21.18	1	21.18	8.95	0,0173
MeJa*HXD	41.27	1	41.27	17.45	0,0031
Error	18.93	8	2.37		
Total	618.3	11			

(*): The effect of each variable was considered significant when p<0.05.

Table 3: Two-Way-ANOVA for MeJa elicitation and HXD addition on IC volumetric AQs production (μmol/L) in hairy root cultures of *R. tinctorum*.

Variable	*SS*	*DF*	*MS*	*F value*	*p-value(*)*
Model	2779175,25	3	926391,75	20.99	0,0004
MeJa	2232831,51	1	2232831,51	50.59	0,0001
HXD	284964,80	1	284964,80	6.46	0,0347
MeJa*HXD	261378,94	1	261378,94	5.92	0,0410
Error	353105,25	8	44138,16		
Total	3132280,50	11			

(*): The effect of each variable was considered significant when p<0.05.

Table 4. Two-Way-ANOVA of MeJa elicitation and HXD addition on EC AQs production (μmol/L) in hairy root cultures of *R. tinctorum*.

Variable	*SS*	*DF*	*MS*	*F value*	*p-value(*)*
Model	28423,67	3	9474,56	57.48	<0,0001
MeJa	10872,12	1	10872,12	65.96	<0,0001
HXD	10405,27	1	10405,27	63.12	<0,0001
MeJa*HXD	7146,27	1	7146,27	43.35	0,0002
Error	1318,71	8	164,84		
Total	29742,38	11			

(*): The effect of each variable was considered significant when p<0.05.

Effectively, EC AQ content reached 120 μmol/L, approximately a 132-fold increase compared with control cultures, and a 10-fold increase compared with MeJa and HXD treatments. AQs accumulated in the second phase were in the order of 80% of the total EC AQs, although the EC AQs represented only 6.71% of the total AQs produced. In Table 2, 3 and 4, the results of the two way ANOVA experiment are summarized.

The addition of a second organic phase for ISPR had a positive effect on transformed root cultures, allowing an increase in the production of AQs. This effect was not observed in the culture of cell suspensions, evidencing the susceptibility of these cultures to the addition of HXD (data not shown). These results are evidence of the different effects that these strategies have on different types of cultures, not only on secondary metabolite accumulation, but also on viability.

Volumetric productivity (Q_p) was compared among suspension and hairy root cultures. MeJa-treated cell cultures (overall time process of 7 days) showed productivities of 80.0 and 74.2 μmol AQs/L.d at MeJa concentrations of 200 and 100 μM, respectively. Q_p in hairy roots treated with MeJa alone yield 46.0 μmol AQs/L.d of AQs, while the combined treatment (MeJa and HXD) showed a productivity level of 74.5 μmol AQs/L.d (considering an overall time process of 25 days). Although hairy root cultures reached higher AQs titers (μmol/L and μmol/gFW) than cell suspensions, the fast growth of cell suspensions resulted in higher or equal productivities than root cultures. However, cell

suspensions may not always be an option. In the case of other secondary metabolites, such as tropane alkaloids, differentiation of the tissue is essential for their production (Cardillo *et al*., 2010).

The results obtained opened the discussion about how different environmental factors influence secondary metabolite production in plant cell cultures. It has been described that 2,4-dichlorophenoxyacetic acid (2,4-D) completely represses the production of AQs in *Morinda citrifolia*, while naphthaleneacetic acid (NAA) induces the biosynthesis of these secondary metabolites (Stalman *et al*., 2003). Schulte *et al*. (1984) observed that the addition of phenoxyacetic acid derivatives and NAA induced AQs production in *R. fruticose* and *R. tinctorum*, respectively. Shin (1989) reported that callus cultures of *R. cordifolia* and *R. akane* treated with NAA showed higher AQs content than those treated with 2,4-D. These reports suggest that AQs biosynthesis is regulated by different kind of auxins, while the role of cytokinins is less significant (Han *et al*., 2001). On the other hand, different elicitors have showed positive effects on AQs accumulation in *R. tinctorum* and *M. elliptica* suspension cultures. Fungal cell wall extracts, jasmonic acid, and salicylic acid triggered AQs production, reaching AQ levels of approximately 3.5 times higher than non-treated *R. tinctorum* cell suspensions (Orbán *et al*., 2008). Jasmonic acid elicitation in *M. elliptica* cell cultures produced a 2-fold increase in AQs accumulation (Chong *et al*., 2005). Perassolo *et al*. (2017) observed a synergistic effect between MeJa elicitation and medium composition. *R. tinctorum* hairy root cultures cultured in half-saline strength Gamborg´s B5 showed higher AQs levels than those grown on WPM, accompanied by an 8.1-fold-increase on AQs release. Finally, MeJa elicitation showed a synergistic effect, resulting in a 2.4-fold increase in AQ content (Perassolo *et al*., 2017).

3.3 Assessment of student learning

Students participating in the course are graduates from different areas: veterinarians, biologists, biochemists, agronomists, etc., constituting a heterogeneous group of students. In general, they have no previous knowledge of *in vitro* culture of plant tissues or its applications. During this two-week course, the students attended lectures regarding general concepts of *in vitro* culture, micropropagation, production of secondary metabolites by *in vitro* culture, plant genetic transformation, metabolic engineering, culture in bioreactors, molecular farming and phytoremediation. They also performed practical work in the laboratory regarding the establishment of *in vitro* cultures (calli and suspensions), the generation of hairy roots by infection of plant explants with *Agrobacterium rhizogenes*, and the transient expression of recombinant proteins by agroinfiltration in leaves of *Nicotiana benthamiana*.

The activities described in this work were complemented by the discussion of the steps to be followed in the laboratory and were also included in a written guide developed by the professors. The discussion was focused on the advantages and disadvantages of the two types of cultures (cell suspensions and transformed roots), the effect of elicitors, the ISPR technique, and the expected results. In addition, the students were introduced into basic knowledge of batch culture, growth kinetics and product formation. Then, all the data obtained were collected in an Excel spreadsheet and the results analyzed and discussed.

Student learning was qualitatively evaluated during the analysis and discussion of the results. In addition to this, students (n=16) were assessed at the end of the course on the basis of the topics presented in both the lectures and practical work. Each of the topics was considered approved if the students obtained a score of 6 or more over a total score of 10. Regarding the topics discussed, when students were evaluated with respect to the advantages and disadvantages of the two different culture systems, 69% of the students approved and the average score reached was 7.5 (n = 16). In contrast, when students were assessed with respect to the effects of elicitation, 100% of them (n = 16) answered correctly, with an average score of 9. Finally, when students were assessed with respect to the ISPR technique, only 55% of them approved (n = 16), with an average score of 6. The general approval of the course was 100%, with an average score of 8. These results may be a consequence of how topics were addressed. Elicitation was more deeply discussed in lectures than ISPR. In fact, the latter was presented as a complement of the former. Regarding the culture systems, this could be attributed to the fact that the choice of hairy roots over cell suspensions depends on the specific case, and developing criteria needs more time of work. In this sense, understanding growth kinetics and culture systems demands more calculation practice. Finally, considering the heterogeneous background of the students, it was expected that some concepts were more difficult to be fully understood for those students

who had no experience in the field. Nevertheless, when students were requested to evaluate themselves about the knowledge acquired during this period of learning, all of them gave positive feedback, stating that the practical work contributed to a better understanding of the subject.

Conclusions

The results of the assessment of student learning showed that the students were able to analyze and estimate the parameters of the batch cultures of cell suspensions and transformed roots and to compare the productivity of each system. In addition, they were able to assess the positive effect of MeJa addition on AQs production in both types of culture and on AQs release in hairy roots. Students were also able to conclude that the addition of HXD as a second phase for the ISPR technique was useful and allowed improving the productivity of transformed root cultures.

The evaluation of the results allowed the students to reinforce their practical knowledge on the topics addressed in the lectures on bioprocesses with plant cultures and to acquire practice in the management and optimization of *in vitro* cultures for the production of secondary metabolites.

Considering the performance of the students when they were assessed, we conclude that we should discuss ISPR with more detail, in order to improve the knowledge of the students in the topic and to incorporate lectures with practical exercises on growth kinetics and product formation in batch culture.

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