Enhancement of anthraquinone production and release by combination of culture medium selection and methyl jasmonate elicitation in hairy root cultures of *Rubia tinctorum*

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ABSTRACT

Anthraquinones (AQs) are secondary metabolites widely distributed in nature. Interesting applications of plant extracts containing AQs include the treatment of Hepatitis C and cancer. Plant *in vitro* culture is an alternative for producing plant-derived pharmaceuticals in controlled conditions and with low environmental impact. Moreover, it allows the application of different strategies for enhancing secondary metabolite production. Hairy root cultures, obtained after *Agrobacterium rhizogenes* infection, are able to produce high amounts of secondary metabolites at high growth rates. In this work, growth kinetics of hairy root cultures of *Rubia tinctorum* and AQ production were evaluated in two different culture media, Gamborg B5 with half of the saline strength ($B5_{1/2}$) and Lloyd & Mc Cown's Woody Plant Medium (WPM). Although WPM allowed higher biomass production (58.6% higher) than $B5_{1/2}$, specific AQ production was higher in $B5_{1/2}$ (between 1.2 and 2.1 fold increases from day 21 to the end of the experiment). Moreover, AQ release to the culture medium was observed in $B5_{1/2}$ (~10% of total AQs). The different performance of hairy roots in these culture media may be due to a limiting nutrient (other than carbon source) in $B5_{1/2}$. Elicitation in $B5_{1/2}$ with methyl jasmonate (100 μM) resulted in a massive accumulation of intracellular (between 1.5 and 2.4-fold increases) and also extracellular AQs (up to 8.1 fold-increase compared with control at 4 days post-elicitation), which could ease AQ purification. These results prove the usefulness of combining different approaches to enhance secondary metabolite accumulation in plant *in vitro* cultures, in order to develop an optimized productive process.

1. Introduction

Plant kingdom has had a central role in human lives since ancient times (Dushenkov and Raskin, 2008; Samuelsson and Bohlin, 2010). According to the World Health Organization, traditional medicine, including the use of herbal medicines, is the primary health service in developing countries and has become relevant in the rest of the world (Cechinel-Filho, 2012). Plant-derived drugs accounts for almost the 25% of the pharmaceutical market (Li and Vederas, 2009), and this proportion rises up to 70% for certain applications, such as the treatment of cancer or infectious diseases (Kolewe et al., 2008).

Plant *in vitro* culture has emerged as an useful strategy for producing plant-derived pharmaceuticals due to its multiple advantages (Siva et al., 2012). Since metabolite production is performed under controlled culture conditions, it is not affected by geographic, seasonal or weather conditions, thus allowing a production that meets the requirements of Good Manufacture Practices (GMP). Besides, it avoids the use of land and the extraction from the natural source, which can be endangered (Huang et al., 2009; Rao and Ravishankar, 2002). Hairy root culture is a differentiated *in vitro* culture obtained after infection of plant tissues with *Agrobacterium rhizogenes* (Bulgakov, 2008). These roots exhibit high growth rates without the need of adding growth

regulators, a characteristic high degree of branching, genetic stability and the capability to produce high amounts of secondary metabolites, in some cases even more than the parent tissue (Guillon et al., 2006; Srivastava and Srivastava, 2007). Moreover, *in vitro* culture allows the implementation of different strategies to enhance the accumulation of secondary metabolites, such as media formulation, *in situ* removal, permeabilization and elicitation (Rao and Ravishankar, 2002; Smetanska, 2008). These strategies, together with the development of new bioreactor configurations suitable for this culture, as well as different modes of operation, allow the stable production of secondary metabolites on a large scale (Cardillo et al., 2010).

Anthraquinones (AQs) are secondary metabolites widely distributed in nature that have been traditionally extracted from madder roots (*Rubia tinctorum* L.) and used as dyes since ancient times (Anjusha and Gangaprasad, 2017; Derksen et al., 2003; Duval et al., 2016). Plant extracts containing AQs have been used with different therapeutic applications. Among them, AQs possess photosensitizing properties since the can generate singlet oxygen and/or superoxide anion radical, and have shown efficacy in photodynamic therapy against cancer cells (Comini et al., 2011; Rumie Vittar et al., 2014). They are also useful in the treatment of Hepatitis C (Wang et al., 2011). In this sense, the hydroxyanthraquinone motif has been described as essential for inhibiting NS3 helicase from this virus (Furuta et al., 2015). Except from the AQs present in herbal preparations, production of specific AQs is mainly achieved by chemical synthesis (Bien et al., 2012). However, the development of production processes in accordance with green chemistry is gaining interest. Secondary metabolite production by plant *in vitro* cultures can minimize the use of both energy and toxic reagents, and the generation of contaminants (Clark, 2006; Constable et al., 2007). In this sense, AQ production by plant *in vitro* cultures may contribute to reduce environmental impact by total or partially replacing its production by chemical synthesis. This strategy is particularly interesting for certain sectors, such as food and pharmaceutical industries, where natural production is highly preferred over organic synthesis (Constable et al., 2007).

The aim of this work was to develop a bioprocess for AQ production by the establishment of hairy root cultures of *R. tinctorum*. The performance of these cultures was evaluated in two different culture media: Gamborg B5 (half-saline strength; $B5_{1/2}$) and Lloyd and McCown's Woody Plant (WPM), and their kinetic and production parameters were calculated. After the selection of the most suitable medium, the effects of methyl jasmonate (MeJ) elicitation were analyzed. This compound has been widely used as effective elicitor in different plant species (Kolewe et al., 2008; Krzyzanowska et al., 2012) and both Jasmonic acid and MeJ have been employed to enhance AQ production in *R. tinctorum* L. *in vitro* cultures (Han et al., 2001; Orbán et al., 2008; Perassolo et al., 2011, 2016). The combination of medium selection and MeJ elicitation reported here is of special interest since it causes not only a drastic increase in AQ production but also a massive AQ release to the culture medium, which is relevant for further improvement of this productive process.

2. Materials and methods

2.1. Establishment of hairy roots of Rubia tinctorum

Hairy roots were obtained as described by Cardillo et al. (Cardillo et al., 2013), with some modifications. Young leaves from *in vitro* cultured- *Rubia tinctorum* L. plantlets were excised and placed abaxial side up in 0.8% agar plates. They were wounded with a scalpel loaded with *Agrobaterium rhizogenes* LBA 9402 grown for 48–72 h at 28 °C in YEB plates. After co-culturing for 4 days at 24 °C in darkness, the explants were transferred to Gamborg B5 solid medium, with the addition of sucrose (20 g L^{-1}), ampicillin (1 g L^{-1}) and agar (0.8%). After 2–4 weeks, roots growing at the site of infection were excised and cultured individually. Clone selection was made according to growth

Table 1
Primers used for amplifying different gene fragments of *A.rhizogenes* LBA 9402.

Gene	Primer	Fragment Size (bp)
rolC	Fw: 5′-TAACATGGCTGAAGACGACC-3′	534
	Rv 5′-AAACTTGCACTCGCCATGCC-3′	
ags	Fw: 5′-GGCGTGAGCACCTCATATCCG-3′	347
	Rv: 5′-TTCGAAGCCTTTGCCTGCAAA-3′	
aux1	Fw: 5′-TTCGAAGGAAGCTTGTCAGAA-3′	350
	Rv: 5′-CTTAAATCCGTGTGACCATAG-3′	
virD	Fw: 5′-ATGTCGCAAGGCAGTAAGCCC-3′	438
	Rv: 5′-GGAGTCTTTCAGCATGGAGCAA-3′	

parameters. Liquid cultures were initiated either in Gamborg B5 (half-saline strength; $B5_{1/2}$) and Lloyd and McCown's Woody Plant (WPM) media, both with the addition of sucrose (20 g L^{-1}). Cultures were subcultured every 30–40 days in the same media and grown in 200 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.

2.2. Confirmation of Agrobacterium-mediated transformation

Transformation with *A. rhizogenes* was confirmed by PCR amplification of *rolC*, *aux1* and *ags* genes in genomic DNA extracted from hairy roots. Genomic DNA was isolated according to Cardillo et al. (Cardillo et al., 2016). Persistence of *A. rhizogenes* was evaluated by PCR detection of *virD* gene. The sequences of the primers were obtained from (Cardillo et al., 2016) and are given in Table 1.

2.3. Growth kinetics of hairy roots

Growth was assessed in 2 different culture media: Gamborg B5 (half-saline strength; $B5_{1/2}$) and Lloyd and McCown's Woody Plant (WPM), both with the addition of sucrose (20 g L^{-1}). In both cases, the same vitamins were added: thiamine hydrochloride (10 mg L^{-1}), pyridoxine hydrochloride (1 mg L^{-1}), nicotinic acid (1 mg $L^{-1)}$ and myo-inositol (100 mg L^{-1}). The hairy roots employed for these experiments were at least subcultured 5 times in the same medium before the assay was done.

For these experiments, approximately 0.25 g of fresh weighed (g_{FW}) *R. tinctorum* hairy roots were inoculated into 25 mL of fresh medium (either WPM or $B5_{1/2}$) contained in 100 mL Erlenmeyer flasks. Hairy root cultures were incubated in the conditions mentioned above. For both treatments, cultures were sampled at 7, 14, 21, 28, 35 and 42 days of culture. For each time point and each condition, three complete Erlenmeyer flasks were harvested.

2.4. Elicitation with methyl jasmonate (MeJ)

This set of experiments was performed by inoculating approximately 0.25 g $_{FW}$ *R. tinctorum* hairy roots (grown in $B5_{1/2}$ medium for 30 days) into 100 mL Erlenmeyer flasks that contained 25 mL of fresh $B5_{1/2}$. After 14 days of culture, MeJ was added to a set of flasks to a final concentration of 100 μM and dimethyl sulfoxide (DMSO, diluent of MeJ) was also added to another set of flasks. Control experiments (without any additive) were also carried out. For all the treatments, cultures were sampled at 0, 2, 4 and 7 days post elicitation (dpe). For each time point and each condition, three complete Erlenmeyer flasks were harvested.

2.5. Analytical techniques

The FW biomass quantification was performed according to Cardillo et al. (Cardillo et al., 2016). Growth index (GI) was calculated according to the following formula:
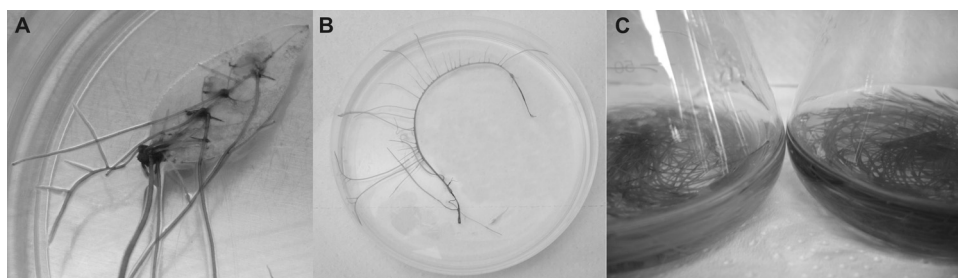
Fig. 1. Hairy roots of *R. tinctorum* obtained after infection *A. rhizogenes* LBA 9402. **A)** Growth from the infection site (each root tip is a clone since it is a consequence of an unique event of transformation). **B)** Growth of an isolated clone in solid culture medium. **C)** Hairy root cultures in liquid medium.
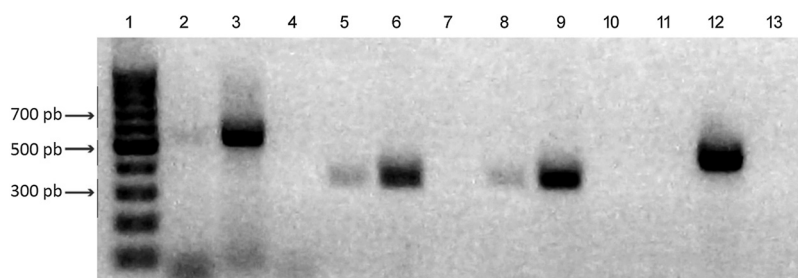

Fig. 2. Agarose gel of PCR products for confirmation of genetic transformation of hairy roots by *A. rhizogenes*. Lane 1: 100–1000 bp ladder; lanes 2, 5, 8 and 11: genomic DNA from hairy roots of *R. tinctorum* was used as template; lanes 3, 6, 9 and 12: *A. rhizogenes* was used as template (colony-PCR); lanes 4, 7, 10 and 13: negative controls. Primer mixes: *rol C* (lanes 2–4); *ags* (lanes 5–7); *aux1* (lanes 8–10) and *virD* (lanes 11–13).
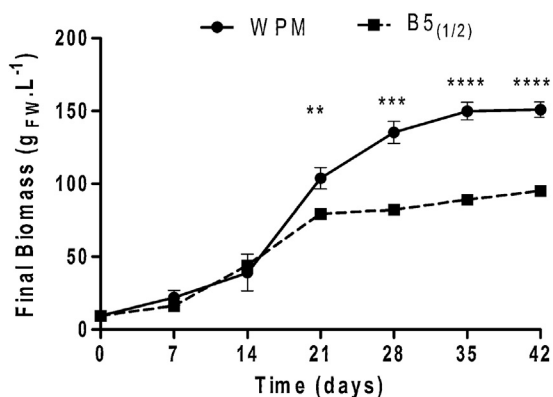

Fig. 3. Time course of growth of hairy roots of *R. tinctorum* grown in WPM and $B5_{1/2}$. Data are depicted as mean ± standard deviation. [**]: Significant differences ($p < 0.01$). [***]: Significant differences ($p < 0.001$). [****]: Significant differences ($p < 0.0001$).

$$GI = (Xf\text{-}Xi)/Xi,$$

where

Xf: final biomass achieved ($g_{FW} L^{-1}$); Xi: initial biomass inoculated ($g_{FW} L^{-1}$)

Hairy root viability was indirectly evaluated by regrowth experiments in solid WPM plates of root tips from samples harvested at 21 days. Sucrose and fructose content in culture media were evaluated

with the anthrone method proposed by van Handel (van Handel, 1967, 1968), after a proper dilution of the sample. Glucose content in culture media was evaluated by using a commercial enzymatic kit for glycaemia determination from Weiner Lab Group (Rosario, Santa Fe, Argentina), after a proper dilution of the sample.

Intracellular AQs content was determined spectrophotometrically at 434 nm, according to Schulte et al. (Schulte et al., 1984). For extracellular AQ content, a sample from culture media was centrifuged at 13000 rpm for 5 min prior to spectrophotometric determination at 434 nm. Insoluble AQ pellet was dissolved in ethanol:water (4:1) at 80 °C and quantified by spectrophotometry at 434 nm. AQ content was calculated using the molar extinction coefficient of alizarin ($5500 M^{-1} cm^{-1}$), the most abundant AQ in *R. tinctorum*.

2.6. Qualitative HPLC analysis

Root samples grown in both WPM and $B5_{1/2}$ after 21 days were grounded to a fine powder in liquid nitrogen and lyophilized. Each dried material was then extracted with ethanol:water (1:1) until exhaustion. These hydro-alcoholic extracts were concentrated to dryness.

HPLC analysis was performed in a Varian Pro Star chromatography apparatus (model 210, series 04171, CA, USA), equipped with an UV–vis detector. A Microsorb-MV column 100-5 C-18 (250 × 4.6 mm i.d.) with an inLine filter (Agilent, CA, USA) was used at 25 °C. Mobile

Table 2
Growth index (GI) for hairy roots grown in WPM and $B5_{1/2}$, calculated at the different harvest times.

Medium	Time (days)					
	7	14	21	28	35	42
WPM[c]	1.26 ± 0.39	3.03 ± 1.48	10.23 ± 0.92[a]	12.83 ± 0.38[a]	14.92 ± 0.78[a]	15.03 ± 0.96[a]
$B5_{1/2}$[d]	0.70 ± 0.07	3.47 ± 0.25	7.44 ± 0.29[b]	7.29 ± 0.85[b]	7.93 ± 0.40[b]	8.40 ± 0.47[b]

Data are expressed as mean ± standard deviation.
[a,b]: Different letters mean significant differences between media ($p < 0.05$) at each harvest time.
[c]: Lloyd and Mc Cown's Woody Plant medium.
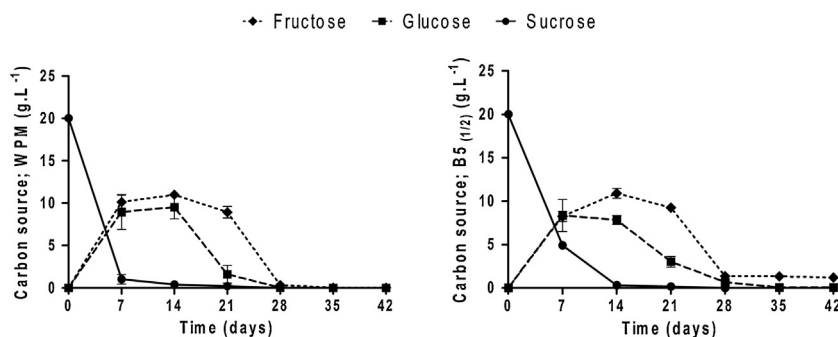[d]: Half saline-strength Gamborg B5 medium.

Fig. 4. Consumption of sucrose (•), glucose (■) and fructose (♦) of hairy root cultures of *R. tinctorum* grown in WPM and B5$_{1/2}$. Data are depicted as mean ± standard deviation.

phase (MP) was a gradient elution of (solvent A) 0.16 M formic acid in ultra-pure water and (solvent B) 0.16 M formic acid in acetonitrile (HPLC grade, Merck, Germany). The composition of MP, at a constant flow (0.4 mL/min), changed from 100% to 21% of solvent A in 65 min, followed by a second ramp to reach 100% B in 5 min, and it stayed fixed during 2 min before returning to 100% A in 2 min. Detector was set at 269 nm. Each dried sample and each reference compound was dissolved in acetonitrile filtered through cellulose (Merck Millipore, Sao Paulo, Brasil) and manual injected (20 μL). Data analysis was performed using the Varian software (Star Chromatography Workstation 6.41, CA, USA).

In addition, a HPLC-DAD-ESI–MS/MS method was performed in an Agilent Technologies 1200 Series system (USA), equipped with a gradient pump (Agilent G1312 B SL Binary), solvent degasser (Agilent G1379 B) and autosampler (Agilent G1367 D SL + WP). The HPLC system was connected to a photodiode array detector (Agilent G1315C Satarlight DAD), and subsequently to a QTOF mass spectrometer (micrOTOF-Q11 Series, Bruker) equipped with an electrospray ionization (ESI) interface. The chromatographic separation was achieved on the same column used above, which was thermostated at 25 °C using a column heater module (Agilent G1316 B). The MP was composed of the two previously used solutions with the same elution gradient at a constant flow of 0.4 mL/min. The injection volume was set at 40 μL. Photodiode array detector was set at 269 nm as preferred wavelengths and the UV–vis spectra were registered from 200 to 600 nm. Mass spectra were recorded in negative ion mode between m/z 50 and 1000. The working conditions for the ionization source were as follows: capillary voltage, 4500 V; nebulizer gas pressure, 4.0 bar; drying gas flow, 8.0 L/min and drying gas temperature, 180 °C. Nitrogen and argon were used as nebulizer and collision gases, respectively. The MS detector was programmed to perform an MS/MS scan of the three most abundant ions, using collision energy of 10.0 eV. Data acquisition and processing were performed using Compass Version 3.1 software and Data Analysis Version 4.0 software, respectively.

Identification was carried out by comparison of the HPLC retention times (t_R) with the corresponding standard compound and co-chromatography with added standards. The t_R values were expressed as means ± SD from three injections for each sample. The following standards were used: alizarin (MP Biomedicals, USA), ruberythric acid and lucidin primeveroside (kindly given by Prof Rob Verpoorte), rubiadin, 2-hydroxy-3-methyl anthraquinone and lucidin ω-methyl ether were purified from *Heterophyllaea pustulata* by J. Marioni (IMBIV-CONICET, Argentina) and identified by means of their spectroscopic data (UV–vis, NMR) in agreement with those found in the literature (Koyama et al., 1991; Nuñez Montoya et al., 2003; Wijnsma and Verpoorte, 1986). In HPLC-DAD-ESI–MS/MS, the identification was also carried out by comparison of the UV–vis spectra and breaks of the ion [M-1] with those obtained for each reference compound.

2.7. Kinetic parameters and experimental yields

Specific growth rate (μ) was calculated as the slope obtained by linear regression from data of ln X = f (t). Doubling time (td), specific substrate consumption rate (q_S), and volumetric productivity (Q_P) were calculated for both processes, as well as biomass to substrate, product to biomass and product to substrate yield coefficients ($Y_{X/S}$, $Y_{P/X}$ and $Y_{P/S}$ respectively). These calculations were made according to the following formulas:

$$\ln X_t = \ln X_0 + \mu * t$$

$$td = \ln2/\mu$$

$$q_S = \mu/Y_{X/S}$$

$$Q_P = P/t$$

$$Y_{X/S} = \Delta X/\Delta S$$

$$Y_{P/X} = \Delta P/\Delta X$$

$$Y_{P/S} = \Delta P/\Delta S,$$

where

t: time (days), S: sucrose (g L^{-1}), P: total AQs (μmol L^{-1}), X: biomass of hairy roots (g$_{FW}$ L^{-1}).

2.8. Statistical analysis

Significance of treatment effects was determined by using Student's test or ANOVA followed by Tukey's Test. The software used for these analyses was InfoStat 2010 Version (Di Rienzo et al., 2010). All experimental data were expressed as mean ± SD of three independent replications.

2.9. Chemicals

Except from where stated, all the chemicals used in this work were of analytical grade and purchased from Sigma Aldrich Chemical Co (St. Louis, MO, USA).

3. Results and discussion

3.1. Establishment of hairy roots of Rubia tinctorum

Hairy roots were obtained after infection with *A. rhizogenes* LBA 9402 (Fig. 1), which is considered as an hypervirulent strain, among others (Makhzoum et al., 2013). T-DNA integration was confirmed by PCR amplification of fragments from *rolC*, *ags* and *aux1* genes, which are present within the T-DNA (Fig. 2). The occurrence of *A. rhizogenes* contaminants in plant DNA samples was checked through PCR ampli-
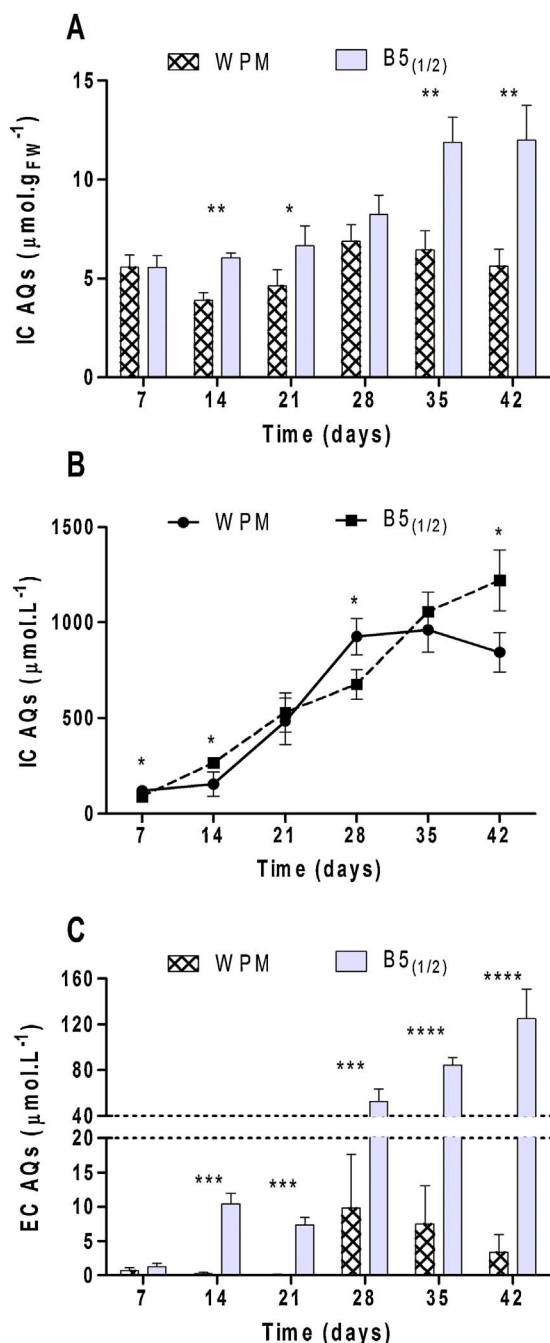
Fig. 5. AQ production by hairy roots of *R. tinctorum* grown in WPM and $B5_{1/2}$: A) specific intracellular AQ production (expressed as μmol per gram of FW biomass); B) volumetric intracellular AQ production (expressed as μmol per liter of culture medium); C) volumetric extracellular AQ production. Data are depicted as mean ± standard deviation. (*): Significant differences ($p < 0.05$). (**): Significant differences ($p < 0.01$). (***): Significant differences ($p < 0.001$). (****): Significant differences ($p < 0.0001$).

fication of the *virD* gene (located outside the T-DNA) and their absence was confirmed.

One of the obtained clones was chosen to assess the performance of growth and AQ production in two different culture media, Gamborg B5 (half-saline strength; $B5_{1/2}$) and Lloyd and McCown's Woody Plant (WPM). Selection of media was done according to previous successful experience with other hairy roots (in the case of $B5_{1/2}$) and suitability of WPM to sustain growth of *R. tinctorum* plants.

3.2. Growth and AQ production kinetics of R. tinctorum hairy roots

Fig. 3 shows biomass production of hairy roots in both culture media. As can be seen, although a similar growth profile was observed in both media, the culture in WPM led to a significantly higher biomass accumulation from day 21 and this difference was maintained until the last harvest day. In consequence, final biomass in $B5_{1/2}$ was 95.1 $g_{FW} L^{-1}$, whereas biomass was a 58.6% higher in WPM ($p < 0.0001$). Comparison of growth index at each harvest time (GI, see Table 2) showed the same behavior, since GI in WPM was significantly higher from day 21 and throughout the rest of the experiment ($p < 0.05$). Biomass yields are comparable to those reported by other authors for hairy roots of other plant species that produce other metabolites, and were cultured in other plant media. In our experiments, biomass showed a 9.7- and a 15.8-fold increases in $B5_{1/2}$ and WPM, respectively, whereas for instance, hairy roots *Panax quinquefolium* L showed a 10-fold increase in biomass content after 8 weeks of culture in one-fourth saline strength Gamborg B5 medium, supplemented with glycine, myoinositol and higher vitamin concentration (Mathur et al., 2010).

Consumption of carbon source is depicted in Fig. 4. In both cases, sucrose was quickly consumed and was almost undetectable from day 14, whereas glucose and fructose showed a concomitant accumulation, reaching maximum levels at 7 and 14 days, respectively. Fructose was more slowly consumed than glucose in WPM (Fig. 4A), but both sugars were barely detected after 28 days. The same profile was observed in $B5_{1/2}$ (Fig. 4B), except that fructose was still detected at 42 days, although at very low levels ($< 1 gL^{-1}$). These results indicate that the carbon source was almost completely utilized in both media. These findings are in accordance with the existence of an invertase bound to plant cell wall, responsible of the hydrolysis of sucrose in the culture medium (Shin et al., 2003).

Intracellular AQ accumulation (IC AQs) was evaluated as specific (per g_{FW} of biomass) and volumetric (per L of culture medium) production. As shown in Fig. 5A, specific IC AQs is significantly higher in $B5_{1/2}$ than in WPM from day14. AQ accumulation reached almost 12 μmol/g_{FW} in $B5_{1/2}$ (2.13 times higher than in WPM; $p < 0.001$), which is 15–20 times higher than that achieved in suspension cultures of *R. tinctorum* (Perassolo et al., 2016).

When analyzing volumetric IC AQ production, this tendency changes (Fig. 5B). The lower specific production achieved by hairy roots in WPM was compensated with the higher biomass production in this media, especially at day 28 when AQ content was even higher in WPM than in $B5_{1/2}$ (36.8%; $p < 0.05$). It is interesting to note that, whereas AQ production was completely growth-associated in WPM, it showed a dual behavior in $B5_{1/2}$: a first phase were AQ production was growth associated, and a second phase where AQs were still being produced while the stationary phase of growth had already been achieved.

Fig. 6 shows the analysis of IC AQ profiles from roots cultured in WPM and $B5_{1/2}$ by HPLC-UV. In both cases, the only AQs identified were ruberythric acid (β-2-alizarin primeveroside) and lucidin primeveroside. The presence of these AQs was corroborated by HPLC-DAD-ESI–MS/MS. Table 3 shows the [M-1] peak, chemical rupture and UV–vis spectral data for each standard. Data from the identified compounds isolated from root samples grown in both $B5_{1/2}$ and WPM are depicted in Table 4. Further analyses should be performed to fully identify all the different AQs produced in both media.

AQ release to the culture media was an interesting phenomenon observed in $B5_{1/2}$ (Fig. 5C). While extracellular AQs (EC AQs) were almost undetectable in WPM, extracellular AQ accumulation in $B5_{1/2}$ increased during the time of the experiment. Although it represents a small proportion of total AQ content (~10%), this finding could be useful for AQ extraction and purification. On the other hand, root tips obtained from both conditions were able to grow in a similar way in solid plates (data not shown). Therefore, it is possible to assume that
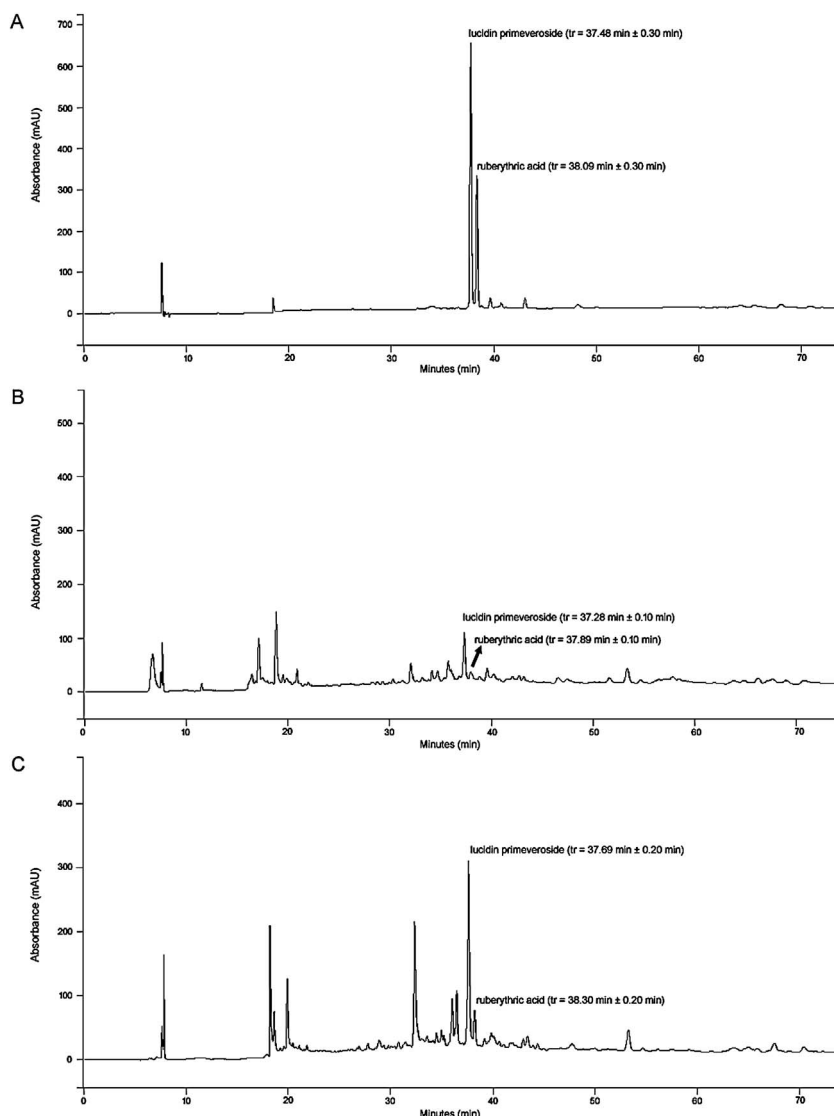
Fig. 6. IC AQ profile from hairy roots grown for 21 d in the different culture media analyzed by HPLC-UV. **A**) Chromatogram of Ruberythric acid and Lucidin primeveroside standards. **B**) Chromatogram from samples obtained from hairy roots cultured in B5$_{1/2}$. **C**) Chromatogram from samples obtained from hairy roots cultured in WPM.

Table 3
HPLC-DAD- ESI–MS/MS data of standard compounds analyzed.

Standard compounds	HPLC parameters			
	tR (min)	[M-1]	MS/MS	UV–vis (nm) experimental
lucidine primeveroside	40.2 ± 0.1	563	311, 269	265, 407 [a]
ruberythric acid	40.4 ± 0.2	533	239	260, 334, 415 [a]
alizarin	56.2 ±	239	211	253, 429 [a]
rubiadin	65.8 ±	253	225	246, 279, 410 [b]
2-OH-3-Me AQ	60.2 ±	237	–	276, 334, 382 [b]
lucidin ω-methyl ether	61.9 ±	283	251	281, 341 (sh), 412 [c]

[a] In accordance with (Cuoco et al., 2011).
[b] In accordance with (Núñez Montoya, 2002).
[c] In accordance with (Koyama et al., 1991).

AQ release was not a consequence of tissue damage or loss of viability.

Kinetic parameters and experimental yields are depicted in Table 5. As can be seen, maximum specific growth rate (μ$_{MAX}$) was slightly higher in WPM than that in B5$_{1/2}$, which is directly related to the higher biomass content achieved in WPM, and therefore, a higher biomass to

substrate yield (Y$_{X/S}$). Specific growth rates and doubling times are comparable to those reported for other species in different culture media (Dhakulkar et al., 2005; Kim et al., 2003). In our experiments, although the same amount of carbon source was added to both media, hairy roots grown in B5$_{1/2}$ were unable to reach the same biomass accumulation. This might be due to the fact that other nutrient, apart from sucrose, is limiting.

Regarding the carbon source, it was completely consumed in both culture media, thus resulting in a higher specific substrate consumption rate (q$_S$) in B5$_{1/2}$ than in WPM (same amount of consumed sucrose by lower amounts of biomass). This could indicate that the carbon from sucrose that was not used for biomass production in B5$_{1/2}$, was destined to AQ biosynthesis. This hypothesis is consistent with the higher product to substrate yield coefficient (Y$_{P/S}$) observed in this culture medium.

Volumetric productivity (Q$_P$) was higher in WPM (Q$_{PMAX}$ achieved at 28 days), due to the higher biomass accumulation that was observed in this culture medium. Nevertheless, a similar productivity was achieved in B5$_{1/2}$ at 35 days of culture.

One possible explanation for the behavior of biomass and AQ production in B5$_{1/2}$ is the limitation of some nutrients when compared with WPM. The higher ratio of sucrose to other nutrients (phosphorus,

Table 4

AQs identified in root samples of *R. tinctorum*, cultivated in both WPM and B5$_{1/2}$ by using HPLC-DAD- ESI–MS/MS.

Identified compounds	WPM			B5$_{1/2}$		
	t_R (min)	[M-1] MS/MS	UV-V	t_R (min)	[M-1] MS/MS	UV-V
Lucidine primeveroside	40.2 ± 0.1	[563], 311, 269	265, 405	40.1 ± 0.1	[563], 311, 269	266, 403
Ruberythric acid	40.5 ± 0.1	[533], 239	260, 278 (sh), 326, 412	40.6 ± 0.2	[533], 239	260, 278 (sh), 326, 413

Table 5

Kinetic parameters and experimental yield coefficients of hairy roots grown in WPM and Gamborg B5$_{1/2}$ media.

Parameter	WPM	B5$_{1/2}$
μ maximum (d^{-1})	0.113	0.093
td (d)	6.13	7.45
Final biomass (g_{FW} L^{-1})	150.9	95.1
$Y_{X/S}$ (g_{FW} X gS^{-1})	7.55	4.76
$Y_{P/X}$ (μmol AQs $g_{FW}X^{-1}$)	8.9	18.5
$Y_{P/S}$ (μmol AQs gS^{-1})	67.2	88.1
q_S (gS $g_{FW}X^{-1}$ d^{-1})	0.015	0.020
Q_P maximum (μmol AQs L^{-1} d^{-1})	33.4 (28 d)	32.6 (35 d)

sulfur and nitrogen) in B5$_{1/2}$ compared with that in WPM might have resulted in a limitation to biomass formation, with no effect on product accumulation. On the other hand, AQs might have been produced as a consequence of the low nutrient content in B5$_{1/2}$.

Other authors have explored different strategies for AQ production. Sato and coworkers evaluated sucrose concentration and phytohormone addition in MS medium and found that AQ content increased with higher sucrose concentration, and that IAA addition resulted in both the highest growth rate and AQ content, but no release to the culture medium was reported (Sato et al., 1991). Regarding medium composition, it was reported that higher AQ yields were obtained by using fructose and nitrate as the only carbon and nitrogen sources, respectively, in modified Murashige & Skoog Medium (MS), and that AQ release was induced by oxygen starvation (Kino-oka et al., 1994).

As hairy roots grown in B5$_{1/2}$ showed a higher specific AQ production and a little though interesting AQ release to the culture media, elicitation experiments were performed in this culture medium.

3.3. Biomass and AQ production after elicitation with methyl jasmonate (MeJ)

In order to evaluate the effect of MeJ elicitation on hairy roots of *R. tinctorum* grown in Gamborg B5$_{1/2}$, roots grown in this culture medium for 14 days were treated with MeJ (100 μM). In addition, control and DMSO (diluent of MeJ) treatments were set in parallel. As is depicted in Fig. 7A, no significant differences were observed in biomass production among hairy roots under MeJ, DMSO and control treatments during the whole experiment. Analyses of root tips obtained from the different treatments at final harvest time showed no differences in growth in solid media (data not shown).

MeJ elicitation had a deep impact on both intracellular (IC) and extracellular (EC) AQ content. Regarding specific IC AQs (Fig. 7B), addition of MeJ resulted in a significant increase at 2 and 4 days post-elicitation (dpe; 49.9 and 59.3%; $p < 0.01$), and an even stronger response at 7 dpe, where IC AQs showed a 2.4 fold-increase (141.6%; $p < 0.05$). As no differences were observed on biomass production, volumetric IC AQs production showed the same profile as that of specific IC AQ production (data not shown). On the other hand, hairy roots only treated with DMSO showed similar IC AQ values as those of control roots.

The strong effect of MeJ on AQ production was also observed on EC AQ content, with 6.5, 8.1 and 4.9-fold increases compared with control treatment at 2, 4 and 7 dpe (increases of 550, 710 and 390%,

respectively; $p < 0.05$, see Fig. 7C). In this case, AQs were released in such an extent that they were found as both soluble and insoluble fractions. Whereas DMSO had only a little effect on soluble EC AQs at 7 dpe (128%; $p < 0.05$), MeJ treatment resulted in increases at 2, 4 and 7 dpe (143, 262 and 207%, respectively; $p < 0.05$). Quantification of insoluble EC AQ fraction showed that it represented a small proportion of whole EC AQs in DMSO (~7.6% at 7 dpe). However, in the case of MeJ, the insoluble fraction accounted for the 63, 56 and 38% of total EC AQ content, at 2, 4 and 7 dpe respectively. Although DMSO may be contributing to the release effect of MeJ treatment (it is the vehicle for MeJ administration), as it has been used for permeabilization treatments (Rao and Ravishankar, 2002), it is clear that MeJ on its own is responsible for the strong elicitation effects, since significant differences were observed between MeJ and DMSO treatments.

Finally, the effect of MeJ on total AQ content rendered in 1.6, 2.1 and 2.3 fold-increases at 2, 4, and 7 dpe, respectively (increases of 60, 110 and 130%, respectively; $p < 0.05$). Volumetric productivity (Q_P) was compared among treatments (see Table 6). MeJ treatment showed the higher productivity during the whole experiment, reaching its highest value at 7 dpe.

Our results show that MeJ is a potent elicitor of AQ production in hairy roots of *R. tinctorum*.

We have previously shown this effect on suspension cultures of this plant species (Perassolo et al., 2011; Perassolo et al., 2016), and other authors have reported the effects of MeJ on hairy roots of *R. tinctorum* (Han et al., 2001; Nakanishi et al., 2005). However, no release of AQs to the culture medium was reported in these works, whereas a drastic increase in AQ production and a massive release of AQs to the culture media was found in our experiments, as a consequence of the combination of MeJ elicitation and culture medium composition. This finding is particularly interesting, since the isolation and purification of these compounds from the culture medium is easier than extraction from plant tissues. Therefore, this platform becomes more attractive as a competitive and greener alternative to current chemical production.

These results provide new evidence of the potential of combining different strategies for the optimization of secondary metabolite accumulation in plant *in vitro* cultures.

4. Conclusions

In this work, AQ production was evaluated in plant *in vitro* cultures by combination of different metabolic strategies. In this way, hairy root cultures of *R. tinctorum* were successfully established by infection with *A. rhizogenes* LBA 9402. Growth kinetics and production parameters of these roots were completely different in the two culture media assayed, Gamborg B5$_{1/2}$ and WPM. These differences may be due to a limiting nutrient (other than carbon source) in Gamborg B5$_{1/2}$, and also a consequence of the low saline strength in Gamborg B5$_{1/2}$, compared with WPM. The higher specific AQ production of hairy roots in B5$_{1/2}$, combined with MeJ elicitation (100 μM), resulted not only in a strong enhancement of AQ accumulation, but also a massive release of AQs to the culture medium. These findings are of special interest since purification from the culture medium is easier than that from plant tissues, thus increasing overall yields and reducing the costs of the whole process. Nevertheless, further improvement of the process is desirable, and this can be achieved by combining elicitors or by
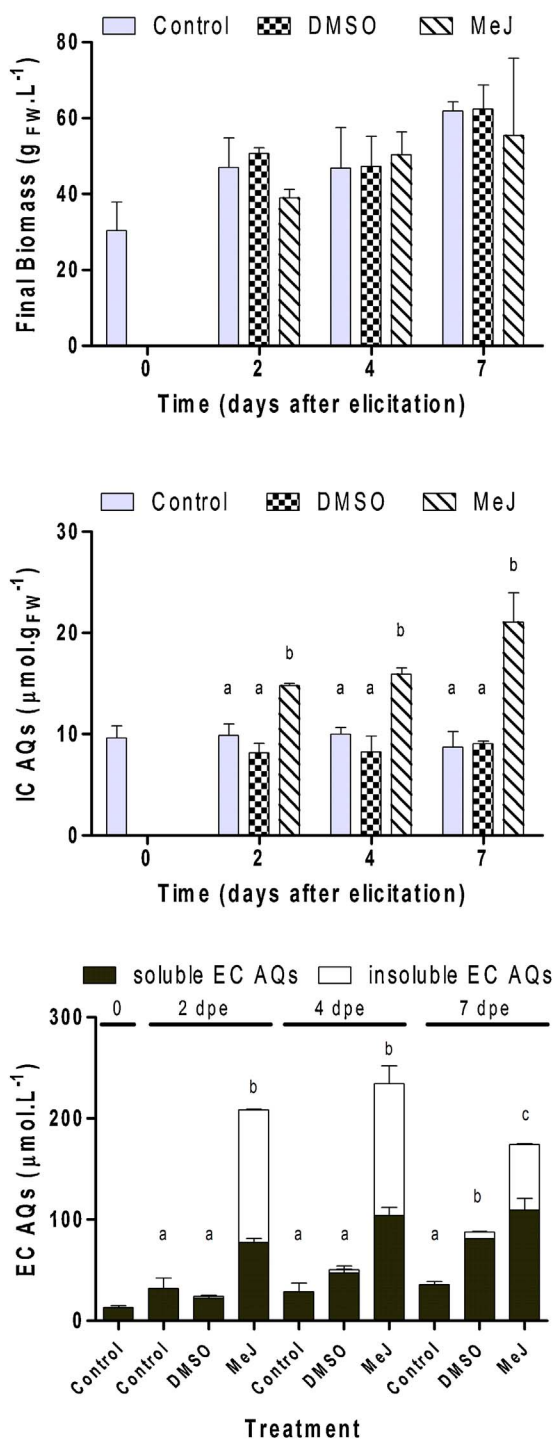
Table 6

Anthraquinone productivity among Control, Dimethyl sulfoxide (DMSO) and Methyl jasmonate (MeJ) treatments in Gamborg B5$_{1/2}$ medium, calculated at the different harvest times.

Treatment	Productivity (μmol L^{-1} d^{-1})		
	t = 2 dpe	t = 4 dpe	t = 7 dpe
Control	31.2 ± 8.0[a]	27.3 ± 4.7[a]	27.5 ± 5.3[a]
DMSO	27.2 ± 3.3[a]	24.9 ± 7.6[a]	31.0 ± 3.5[a]
MeJ	49.0 ± 1.3[b]	57.4 ± 7.0[b]	62.4 ± 12.3[b]

Data are expressed as mean ± standard deviation (n = 3).

[a,b]: Different letters represent significant differences between treatments ($p < 0.05$) at each harvest time.

strategies, a competitive and greener process could be developed, thus becoming an alternative to current chemical production. In this sense, *in vitro* production of AQs can completely replace chemical production in some specific cases, or can provide the basic skeleton to produce a variety of AQ-derived compounds by minor chemical modifications, thus reducing the use and generation of toxic compounds.

In conclusion, the results obtained in this work represent an attractive proof of the usefulness of the combination of different approaches to enhance secondary metabolite accumulation in plant *in vitro* cultures, in order to develop an optimized process for the production of plant secondary metabolites.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Fig. 7. A) Biomass of hairy roots of *R. tinctorum* grown in B5$_{1/2}$ after the different treatments (control, DMSO and 100 μM MeJ). **B**) Specific intracellular AQ production (expressed as μmol per gram of FW biomass) of hairy roots after elicitation and control treatments. **C**) Volumetric extracellular AQ production, showing the different proportion of soluble and soluble fractions of AQs in the different treatments performed on *R. tinctorum* hairy roots. Data are depicted as mean ± standard deviation (n = 3). [a,b,c] Different letters indicate significant differences among treatments ($p < 0.05$).

applying *in situ* removal strategies, in order to increase productivity. After defining the most appropriate combination of strategies, scaling-up in a suitable bioreactor and optimization of the fermentative process could increase yields. For instance, a continuous culture of these hairy roots could be established in mist bioreactors, and while fresh culture medium is periodically added, exhausted medium with secreted AQ could be removed. By combining metabolic and bioreactor engineering

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