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ORIGINAL ARTICLE

Synergistic effect of methyl jasmonate and cyclodextrins on anthraquinone accumulation in cell suspension cultures of *Morinda citrifolia* and *Rubia tinctorum*

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Abstract Plant in vitro culture is a platform for producing secondary metabolites that combines safety, quality and low environmental impact. Besides, it is possible to increase the accumulation of these compounds by different strategies, such as elicitation. In this work, we analyzed the effects of the combination of methyl jasmonate (MeJ) and two cyclodextrins (CDs) on the production of anthraquinones (AQs) in cell cultures of Rubiaceae (*Morinda citrifolia* and *Rubia tinctorum*). These secondary metabolites have been traditionally used as dyes and have interesting therapeutic applications. The experiments were designed according to a full factorial design of two factors (MeJ and a CD) in two levels (0 and 0.1 mM for MeJ, and 0 and 20 mM of the CD). MeJ and (2-hydroxypropyl)-β-cyclodextrin (HPCD) synergistically increased intracellular AQ content in suspension cultures of *R. tinctorum*, and, to a lesser extent, in suspension cultures of *M. citrifolia*. Combination of MeJ with another CD, 2-methyl-β-cyclodextrin, led to a more intense and later increase in AQ content in cell cultures of *R. tinctorum* when compared to MeJ–HPCD treatment. However, the combination of CD and MeJ failed to induce a drastic AQ release to the culture media. Nevertheless, our results show that combination of strategies (using a CD and MeJ) was successful to increase secondary metabolite accumulation in suspension cultures. To our knowledge, this is the first report of synergistic effect of MeJ and CD on AQ accumulation in plant in vitro cultures.

Keywords Anthraquinones · Cyclodextrin · Elicitation · Methyl jasmonate · Plant in vitro culture · Rubiaceae

Introduction

Anthraquinones (AQs) are secondary metabolites widely distributed in nature, as they are produced by plants, bacteria, fungi, lichens and even animals (in *Dactylopius coccus*). Among plants, AQs can be isolated from species of Fabaceae (Leguminosae), Polygonaceae, Rhamnaceae, Gesneriaceae, Liliaceae and Rubiaceae (Han et al. 2001; Samuelsson 1999). These secondary metabolites have been traditionally used as dyes, and are employed as additives in the paper and pulp industry (Butterworth et al. 2001). In addition, they exhibit interesting therapeutic applications, such as antiviral (against Hepatitis C virus), and as photosensitizers of cancer cells (Comini et al. 2011; Rumie Vittar et al. 2014; Wang et al. 2011). Although nowadays AQs are almost completely produced by chemical synthesis, there is a growing interest in developing processes that minimize the use of both energy and toxic reagents, and the generation of contaminants (Clark 2006; Constable et al. 2007).

Plant in vitro culture has emerged as a safe and low cost alternative for the production of bioactive compounds. Besides, it allows the implementation of Good

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Manufacture Practices (GMP) and avoids the extraction of the secondary metabolites from the natural source, which could become endangered (Huang et al. 2009; Rao and Ravishankar 2002). Although the accumulation of secondary metabolites may be low, in vitro culture enables the application of different strategies for increasing the production these compounds and also their release to the culture media, which facilitates their purification (Hanchinal et al. 2008; Smetanska 2008). Elicitation has been extensively used for these purposes, since elicitors are biotic or abiotic factors able to trigger a defense response that usually involves the induction of secondary metabolite accumulation (Vasconsuelo and Boland 2007; Zhao et al. 2005). Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJ) are naturally produced compounds that are involved in plant defense responses and have been widely used as effective elicitors (Kolewe et al. 2008; Krzyzanowska et al. 2012). In this sense, we have demonstrated in a previous work that MeJ induced AQs biosynthesis in suspension cultures of *Rubia tinctorum* and that this effect was mediated by H_2O_2 (Perassolo et al. 2011a).

Cyclodextrins (CD) are cyclic oligosaccharides derived from starch with multiple applications in cosmetics and textile, pharmaceutical and food industry due to its interesting properties: increased solubility of non-polar compounds, increased stability, protection against degradation caused by heat, oxidation and UV radiation (Singh et al. 2002). They have been used in biotransformation to increase substrate solubility and to enhance secondary metabolite accumulation and release to the culture medium (Bru et al. 2006; Sabater-Jara and Pedreño 2013; van Uden et al. 1994). In recent works, it has been shown that the combined addition of MeJ and CD resulted in an increased production of resveratrol in grapevine cell cultures (Belchi-Navarro et al. 2012; Lijavetzky et al. 2008; Martinez-Esteso et al. 2011), as well as phytosterols in hairy roots of *Solanum tuberosum* (Komaraiah et al. 2003), sesquiterpenes and phytosterols in cell cultures of *Capsicum annum* (Sabater-Jara et al. 2010), terpenoid indole alkaloids in cell cultures of *Catharanthus roseus* (Almagro et al. 2014), artemisinin in *Artemisia annua* (Durante et al. 2011), and taxanes in cell cultures of *Taxus* × *media* (Sabater-Jara et al. 2014).

The aim of this work was to evaluate the effects of two CD, (2-hydroxypropyl)-β-cyclodextrin (HPCD) and 2-methyl-β-cyclodextrin (MCD) in combination with MeJ on AQ production in suspension cultures of two species of Rubiaceae, *R. tinctorum* and *Morinda citrifolia*, by performing experiments with a two factorial design.

Materials and methods

Cell suspension cultures

Rubia tinctorum L. and *Morinda citrifolia* suspension cultures were a kind gift of Dr. Rob Verpoorte (Leiden University, The Netherlands). Cells were cultured in Gamborǵs B5 medium, supplemented with 20 g L^{-1} sucrose, pH: 5.75–5.80. In the case of *R. tinctorum* cells, the growth regulators added were: 2 mg L^{-1} 2,4-dichlorophenoxyacetic acid, 0.5 mg L^{-1} 1-naphthaleneacetic acid, 0.5 mg L^{-1} indole acetic acid, and 0.2 mg L^{-1} kinetin. For *M. citrifolia*, 1.86 mg L^{-1}1-naphthaleneacetic acid was added. 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. Sub culturing was carried out every 7–10 days, using a threefold dilution of cells. All the experiments were carried out with cell suspensions cultured as mentioned.

Full factorial experiment (2^2) for MeJ and HPCD addition in cell suspension cultures of *R. tinctorum* and *M. citrifolia*

In these experiments, approximately 2.5 g of fresh weighed (FW) *R. tinctorum* cells were inoculated into 25 mL of B5 fresh medium (supplemented as mentioned above) with or without the addition of 20 mM (28 g L^{-1}) HPCD, contained in 100 mL Erlenmeyer flasks. Suspension cultures were incubated for 3 days in the conditions mentioned above, and after this time period, each set of cultures (B5 and B5-HPCD) were divided into 2 groups, and one group of each set was treated with 0.1 mM MeJ. In this way, four different experiments were performed, rendering a full factorial design of two factors (HPCD and MeJ) in two levels (0 and 20 mM for HPCD; 0 and 0.1 mM MeJ.

Table 1 Codification of the factors and full factorial design (2^2) for *M. citrifolia* and *R. tinctorum* cell suspension cultures

Experiment	Concentration (mM)		Codification level	
	CD[a]	MeJ	CD[a]	MeJ
1	0	0	0	0
2	20	0	1	0
3	0	0.1	0	1
4	20	0.1	1	1

[a] *CD* cyclodextrin (either HPCD or MCD)

Codification of the factors and the full factorial design are shown in Table 1. The resulting experiments were: HPCD 0–MeJ 0 (B5 medium alone), HPCD 1–MeJ 0 (B5 medium with 20 mM HPCD), HPCD 0–MeJ 1 (B5 medium with 0.1 mM MeJ), and HPCD 1–MeJ 1 (B5 medium with both 20 mM HPCD and 0.1 mM MeJ). For all treatments, samples were taken at 3 (prior to elicitation with MeJ), 6 and 8 days of culture. Each experiment was carried out in quadruplicate.

The same set of experiments was performed with *M. citrifolia* suspension cultures, but in this case, the initial biomass was 2 g (FW) in 25 ml of B5 medium (supplemented as mentioned before). Each experiment was carried out in triplicate.

Full factorial experiment (2^2) for MeJ and MCD addition in cell suspension cultures of *R. tinctorum*

In this case, approximately 2.5 g of FW *R. tinctorum* cells were inoculated into 25 mL of B5 fresh medium with or without the addition of 20 mM MCD (26.2 g L^{-1}), contained in 100 mL Erlenmeyer flasks and cultured for 3 days, when MeJ was added. As before, four different experiments were performed, rendering a full factorial design of two factors (MCD and MeJ) in two levels (0 and 20 mM for MCD; 0 and 0.1 mM MeJ). The resulting experiments were: MCD 0–MeJ 0 (B5 medium alone), MCD 1–MeJ 0 (B5 medium with 20 mM MCD), MCD 0–MeJ 1 (B5 medium with 0.1 mM MeJ), and MCD 1–MeJ 1 (B5 medium with both 20 mM MCD and 0.1 mM MeJ). For all treatments, samples were taken at 6 and 8 days of culture (3 and 5 days post elicitation; dpe). Each experiment was carried out in triplicate.

Analytical techniques

For FW determination, plant cells were harvested, washed once with distilled water and filtrated under gentle vacuum for 2 min. Dried weight (DW) was determined after drying cells at 60 °C until constant weight was achieved. No significant differences in the water content of the cells were found between treatments. DW represents a 4.95 % of the recorded FW.

Cell viability was evaluated by the Evans Blue exclusion test, as mentioned by Smith et al. (1982). Briefly, cell samples were incubated with the dye for 15 min, and after 3 washes with distilled water, the dye was extracted 3 times at 50 °C with 1 % sodium dodecyl sulfate (SDS) in methanol:water (1:1, v/v). The fractions were collected together and the absorbance was measured at 600 nm. Results were expressed as relative units (RU) with respect to control values (1 RU).

AQs content was determined spectrophotometrically, according to Schulte et al. (Schulte et al. 1984), and expressed as μmol per gram of DW cells (specific production) and μmol per liter of culture medium (volumetric production). The same relative changes were observed in response to the different treatments when AQ levels were expressed either as μmol gDW^{-1} or as μmol gFW^{-1}.

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 ($p = 0.05$). In case of significant interaction, the analysis was performed at each level of each variable. 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.

Chemicals

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

Results and discussion

Effects of the addition of HPCD and MeJ on *M. citrifolia* cell cultures

Figure 1a shows the biomass accumulation of *M. citrifolia* cell cultures after the combined treatment with MeJ and HPCD. Prior to elicitation, *M. citrifolia* cell cultures treated with HPCD (20 mM) showed no significant differences in biomass production after 3 days of culture when compared to untreated cultures. Elicitation with 0.1 mM MeJ of both HPCD-treated and non-treated cultures resulted in four different experiments (see Table 1). After 6 days of culture (3 dpe), the statistical analysis showed a significant interaction between HPCD and MeJ ($p = 0.0271$), thus meaning that the presence of one factor affects the response to the other. Therefore, the effects of each factor were analyzed at each level of the other (simple effects). The addition of HPCD increased biomass accumulation only at level 0 of MeJ (14.7 %; $p < 0.05$; comparison between HPCD 0–MeJ 0 and HPCD 1–MeJ 0), whereas the addition of MeJ resulted in lower biomass production (16.2 % lower; $p < 0.05$) only at level 1 of HPCD (comparison between HPCD 1–MeJ 0 and HPCD 1–MeJ 1). The same behavior was observed after 8 days of culture (5 dpe), when the interaction between MeJ and HPCD was again

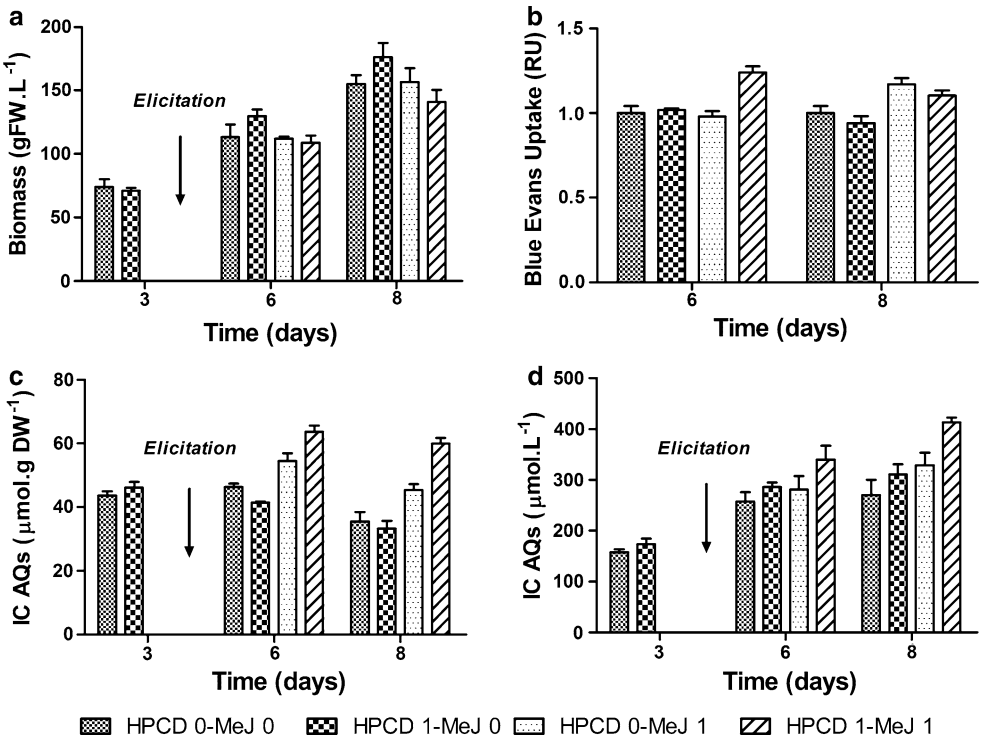

Fig. 1 Results for cell suspension cultures of *M. citrifolia* after the different treatments according to the full factorial design (2^2) with HPCD and MeJ: **a** Biomass accumulation; **b** Cell viability; **c** Specific AQ production (expressed as µmol gDW^{-1}); **d** Volumetric AQ production (expressed as µmol L^{-1}). Data correspond to mean values of 3 replicates and *error bars* indicate SD

significant ($p = 0.0113$). As before, HPCD increased biomass production at level 0 of MeJ (13.7 % more in HPCD 1–MeJ 0 when compared to HPCD 0–MeJ 0; $p < 0.05$), whereas elicitation with MeJ resulted in a decrease in growth (20.1 %; $p < 0.05$) only in HPCD-treated cells (comparison between MeJ 0–HPCD 1 and MeJ 1–HPCD 1). Tables with data analysis and interaction plots are included in a supplemental archive (Figure S1 and Tables S1 and S2).

Our results indicate that HPCD has a positive effect on growth when added alone, whereas MeJ has no effect. On the contrary, the combined addition of MeJ and HPCD has a negative effect on biomass accumulation. Treatments with MeJ, CD and both have been reported to exert different effects on growth. For instance, in *Vitis vinifera* cell cultures, a negative effect on growth was recorded after treatment with MeJ and both MeJ and CDs, whereas CDs alone had no effect (Belchi-Navarro et al. 2012; Lijavetzky et al. 2008). On the other hand, treatment with MeJ and CD decreased cell growth in cell cultures of *S. lycopersicum* cv Micro Tom, even more than CD alone (Briceño et al. 2012). Finally, in cell cultures of *A. annua*, treatment with MeJ, various CDs (HPCD, DIMEB and native β-cyclodextrin) and MeJ combined with CDs did not affect cell growth (Durante et al. 2011). Although cell culture conditions and elicitation procedures are not the same, it seems that MeJ and CD have different effects depending on plant species.

We also evaluated the effect of the treatments on cell viability by performing an exclusion test with Evans Blue. In this way, a high Evans Blue uptake (expressed as Relative Units to HPCD 0 –MeJ 0 treatment) represents low cell viability. These results are depicted in Fig. 1b. At 6 days of culture (3 dpe), the interaction between MeJ and HPCD turned out to be significant ($p = 0.0057$), so simple effects were analyzed. In this case, the addition of HPCD alone had no effect on cell viability (comparison between MeJ 0–HPCD 0 and MeJ 0–HPCD 1), and the same effect was observed when MeJ was added alone (comparison MeJ 0–HPCD 0 and MeJ 1–HPCD 0). On the other hand, an increase in Evans Blue uptake (and therefore, lower cell viability) was recorded when both factors were present (comparison between MeJ 0–HPCD 1 and MeJ 1–HPCD 1, and between MeJ 1–HPCD 0 and MeJ 1–HPCD 1). At 8 days of culture (5 dpe), the interaction between factors was not significant, so principal effects of each factor were analyzed. In this situation, only the addition of MeJ had an adverse effect on cell viability (17.3 % more uptake; $p < 0.05$). Other authors have reported no effects of elicitor treatment on cell viability (Belchi-Navarro et al. 2012; Briceño et al. 2012; Durante et al. 2011; Lijavetzky et al. 2008).

Intracellular AQ accumulation was evaluated in terms of specific production (µmol gDW^{-1} of biomass; Fig. 1c), and volumetric production (µmol L^{-1} of culture medium; Fig. 1d).

As shown in Fig. 1c, addition of HPCD has no effect on AQ accumulation (μmol gDW^{-1}) prior to elicitation. On the other hand, the combination of MeJ and HPCD rendered a significant interaction at 6 and 8 days of culture ($p = 0.0063$ and $p = 0.0126$, respectively).

The statistical analysis of simple effects is depicted in Tables 2 and 3. At 6 days of culture (3 dpe), an increase in AQ production was observed only when HPCD was added at level 1 of MeJ (16.9 %; $p < 0.05$), but in the case of MeJ, the increase was slight at level 0 of HPCD (17.7 %; $p < 0.05$) and greater at level 1 of HPCD, (54.0 %; $p < 0.05$). The same behavior was observed at 8 days of culture (5 dpe), when the addition of HPCD enhanced AQ accumulation in the presence of MeJ (31.7 %; $p < 0.05$), whereas the addition of MeJ resulted in a 27.9 % increase in AQ production ($p < 0.05$) at level 0 of HPCD and an 80.1 % ($p < 0.05$) in the presence of HPCD. Interaction plots are included in a supplemental archive (Figure S2).

Figure 1d shows the volumetric production of AQs (expressed as μmol L^{-1}) in response to HPCD and MeJ treatment. The addition of HPCD had no effect on AQ content at 3 days of culture (prior to elicitation). At 6 and 8 days of culture (3 and 5 dpe), no significant interaction was observed between HPCD and MeJ, thus meaning that there was no synergistic effect between the two factors. Therefore, the principal effects of each of them were evaluated. The addition of HPCD increased AQ content at 6 and 8 days of culture (16.1 and 21.9 % respectively; $p < 0.01$), and the same effect was observed in response to MeJ at both 6 and 8 days of culture (14.4 %; $p < 0.05$ and 27.7 %; $p < 0.01$, respectively).

According to these results, there is a synergistic effect between MeJ and HPCD when the AQ content is expressed per unit of biomass (μmol gDW^{-1}), but it is not detected when the AQ content is expressed per liter of culture medium (μmol L^{-1}). These differences can be attributed to the negative effect of the combined addition of MeJ and HPCD on growth. Therefore, the synergistic effect on AQ production is masked by the lower biomass accumulation when both compounds are present.

In other species, treatment with CD alone caused the release of secondary metabolites to the culture medium (Bru et al. 2006; Sabater-Jara and Pedreño 2013). Moreover, the synergistic effect of MeJ and HPCD was previously reported in other species with different CDs, and, in many cases, extracellular accumulation was also observed (Almagro et al. 2014; Belchi-Navarro et al. 2012; Corchete and Bru 2013; Durante et al. 2011; Komaraiah et al. 2003; Lijavetzky et al. 2008; Sabater-Jara et al. 2010, 2014). Although the synergy between MeJ and HPCD was verified, extracellular accumulation of AQ in cell suspension cultures of *M. citrifolia* was not detected under our experimental conditions. On the other hand, MeJ alone had a little effect on AQ accumulation. An intense response to MeJ elicitation has been reported previously in *M. citrifolia* cell cultures (Komaraiah et al. 2005). In that case, however, elicitation was carried out in different experimental conditions. In a previous work, we reported a strong increase in AQ accumulation in *R. tinctorum* suspension cultures after MeJ treatment (Perassolo et al. 2011a). Although both species are highly related, their susceptibility to elicitors might be different (Vasconsuelo and Boland 2007).

Effects of the addition of HPCD and MeJ on *R. tinctorum* cell cultures

In order to continue the analysis of the effects of HPCD and MeJ on AQ production, we performed the same experiments (following a 2^2 full factorial design) on *R. tinctorum* (see Table 1).

Since there was no significant differences in biomass production (data not shown), the analysis was focused on AQ production.

Figure 2a shows the intracellular AQ accumulation expressed as μmol gDW^{-1}. HPCD significantly increased AQ accumulation (12.9 %; $p < 0.05$) at 3 days of culture (before elicitation).

At 6 days of culture (3 dpe), since interaction between MeJ and HPCD proved to be significant ($p < 0.0001$), the simple effects of each factor were analyzed (Table 4). When MeJ was 0, the addition of HPCD had no effect on AQ accumulation, but in the presence of MeJ (level 1), AQ production was 66.6 % higher ($p < 0.05$) in HPCD-treated cells (compared to those without HPCD). Regarding MeJ,

Table 2 Simple effects of HPCD and MeJ on intracellular AQ production (expressed as μmol gDW^{-1}) in *M. citrifolia* cell cultures after 6 days of culture, analyzed by Tukey's test (with significant HPCD–MeJ interaction by two-way ANOVA)

	HPCD 0		HPCD 1	
	AQs (μmol gDW^{-1})	Result*	AQs (μmol gDW^{-1})	Result
MeJ 0	46.26	A	41.33	A
MeJ 1	54.45	B	63.63	C

* Different letters represent significant differences among medias by Tukey's test ($p < 0.05$)

Table 3 Simple effects of HPCD and MeJ on intracellular AQ production (expressed as μmol gDW^{-1}) in *M. citrifolia* cell cultures after 8 days of culture, analyzed by Tukey's test (with significant HPCD–MeJ interaction by two-way ANOVA)

	HPCD 0		HPCD 1	
	AQs (μmol gDW^{-1})	Result*	AQs (μmol gDW^{-1})	Result
MeJ 0	35.52	A	33.25	A
MeJ 1	45.45	B	59.87	C

* Different letters represent significant differences among medias by Tukey's test ($p < 0.05$)

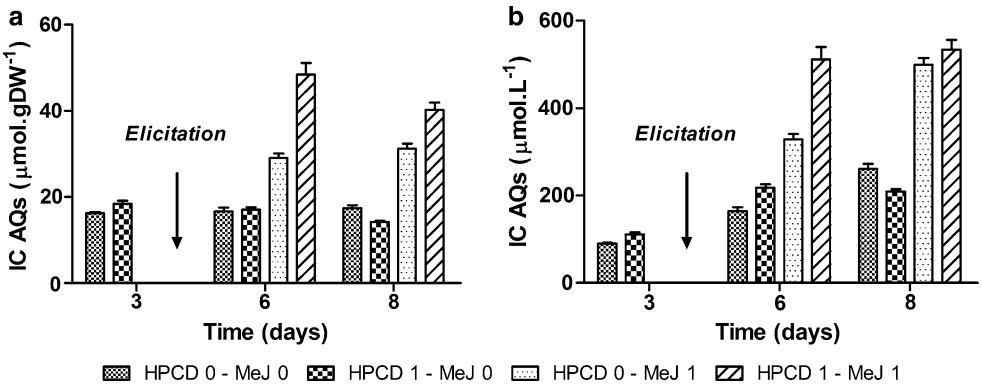

Fig. 2 Intracellular AQ production in cell suspension cultures of *R. tinctorum* in the different experiments of the full factorial design (2^2) with HPCD and MeJ: **a** Specific production (expressed as μmol gDW^{-1}); **b** Volumetric production (expressed as μmol L^{-1}). Data correspond to mean values of 4 replicates and *error bars* indicate SD

Table 4 Simple effects of HPCD and MeJ on intracellular AQ production (expressed as μmol gDW^{-1}) in *R. tinctorum* cell cultures after 6 days of culture, analyzed by Tukey's test (with significant HPCD–MeJ interaction by two-way ANOVA)

	HPCD 0		HPCD 1	
	AQs (μmol gDW^{-1})	Result*	AQs (μmol gDW^{-1})	Result
MeJ 0	16.72	A	17.09	A
MeJ 1	29.08	B	48.48	C

* Different letters represent significant differences among medias by Tukey's test ($p < 0.05$)

it had a positive effect on AQ accumulation at level 0 of HPCD (73.4 %; $p < 0.05$), and even greater at level 1 of HPCD (183.6 %; $p < 0.01$).

The same behavior was observed at 8 days of culture (5 dpe), when the interaction between MeJ and HPCD was extremely significant ($p < 0.0001$). HPCD increased AQ content only when MeJ was present (28.5 %; $p < 0.05$), whereas MeJ promoted AQ production alone (level 0 of HPCD, 80.4 %; $p < 0.05$) and in combination with HPCD (183.9 %; $p < 0.05$). This analysis is shown in Table 5.

When comparing the volumetric AQ production (expressed as μmol L^{-1}; Fig. 2b), there was also a significant interaction between MeJ and HPCD, at both 6 and 8 days of culture ($p = 0.0022$ and $p = 0.0134$, respectively). The

Table 5 Simple effects of HPCD and MeJ on intracellular AQ production (expressed as μmol gDW^{-1}) in *R. tinctorum* cell cultures after 8 days of culture, analyzed by Tukey's test (with significant HPCD–MeJ interaction by two-way ANOVA)

	HPCD 0		HPCD 1	
	AQs (μmol gDW^{-1})	Result*	AQs (μmol gDW^{-1})	Result
MeJ 0	17.38	A	14.19	A
MeJ 1	31.35	B	40.29	C

* Different letters represent significant differences among medias by Tukey's test ($p < 0.05$)

Table 6 Simple effects of HPCD and MeJ on intracellular AQ production (expressed as μmol L^{-1}) in *R. tinctorum* cell cultures after 6 days of culture, analyzed by Tukey's test (with significant HPCD–MeJ interaction by two-way ANOVA)

	HPCD 0		HPCD 1	
	AQs (μmol L^{-1})	Result*	AQs μmol L^{-1}	Result
MeJ 0	164.5	A	218.3	B
MeJ 1	328.8	B	511.3	C

* Different letters represent significant differences among medias by Tukey's test ($p < 0.05$)

Table 7 Simple effects of HPCD and MeJ on intracellular AQ production (expressed as μmol L^{-1}) in *R. tinctorum* cell cultures after 8 days of culture, analyzed by Tukey's test (with significant HPCD–MeJ interaction by two-way ANOVA)

	HPCD 0		HPCD 1	
	AQs (μmol L^{-1})	Result*	AQs μmol L^{-1}	Result
MeJ 0	262.0	B	209.7	A
MeJ 1	499.1	C	533.9	C

* Different letters represent significant differences among medias by Tukey's test ($p < 0.05$)

statistical analyses are depicted in Tables 6 and 7. HPCD alone (level 0 of MeJ) induced AQ production at 6 days of culture (32.7 %; $p < 0.05$) but slightly decreased AQ accumulation at 8 days of culture (20.0 % less, $p < 0.05$). At level 1 of MeJ, HPCD only had a significant effect at 6 days of culture (55.5 % increase; $p < 0.05$). On the other hand, MeJ enhanced AQ production at both 6 and 8 days of culture, without HPCD (99.9 and 90.5 %, respectively; $p < 0.05$), and even more when HPCD was added (134.2 and 154.6 %, respectively; $p < 0.05$). Interaction plots for both specific and volumetric AQ production are included in a supplemental archive (Figures S3 and S4).

Interaction between HPCD and MeJ provoked a significant AQ release to the culture medium (data not shown). However, this effect was not as relevant as that reported by other authors (Almagro et al. 2014; Belchi-Navarro et al. 2012; Corchete and Bru 2013; Durante et al. 2011; Komaraiah et al. 2003; Lijavetzky et al. 2008; Sabater-Jara et al. 2010, 2014), since the extracellular AQ content represented less than 3 % of the total AQs produced. These differences may be a consequence of the different localization of secondary metabolites in plant tissues. Whereas in plant cell cultures of *R. tinctorum* AQs are stored in vacuoles (Bóka et al. 2002), trans-resveratrol is known to accumulate in cell wall of grapevine (Zhang et al. 2011), as well as taxane and related compounds in cell cultures of *Taxus* (Naill et al. 2012).

These results show that HPCD and MeJ have a synergistic effect on AQ production in cell suspension cultures of *R. tinctorum*. This effect was more intense than that registered in *M. citrifolia* cell cultures. Moreover, a slight (although not relevant) AQ release to the culture media was observed only in *R. tinctorum* cultures, whereas no AQs were detected in culture medium of cell culture of *M. citrifolia*.

On the other hand, HPCD and MeJ had no effect on growth of *R. tinctorum* cultures, whereas the combined addition of both of them impaired cell growth in *M. citrifolia*. As was discussed before, this is a new evidence of the different effect of MeJ, CDs and both on biomass accumulation in different plant species.

Effects of the addition of MCD and MeJ on *R. tinctorum* cell cultures

Since the synergistic effect of HPCD and MeJ was more intense and relevant in *R. tinctorum* than in *M. citrifolia*, we continued the analysis of the interaction between CD and MeJ with a new factorial experiment in cell suspension cultures of *R. tinctorum*. In this case, we studied the combined addition of MeJ and methyl-β-cyclodextrin (MCD).

Figure 3a shows the biomass production of *R. tinctorum* cell cultures after the experiment. There was no significant interaction between MeJ and MCD after 6 days of culture, and no significant effects were observed for MCD and MeJ. At 8 days of culture, interaction was significant ($p = 0.0035$), and simple effects of MeJ and MCD were analyzed. MCD addition decreased biomass production at both levels of MeJ (19.9 % at level 0 and 10.2 % at level 1; $p < 0.05$), but MeJ only had effect at level 1 of MCD (9.1 % increase; $p < 0.05$). As can be seen, both compounds had a small (although statistically significant) effect on growth. Statistical analysis is shown in a supplemental archive (Table S3).

It is interesting to note the different effects of the two CDs tested (MCD and HPCD) on *R. tinctorum* cell cultures. Whereas HPCD had no effect, alone or in combination with MeJ, MCD affected cell growth when added alone or in combination with MeJ.

Cell viability was evaluated at the end of the experiment (8 days of culture). As shown in Fig. 3b, MeJ and MCD had a significant interaction ($p = 0.0177$). MCD had an effect only at level 0 of MeJ (15 % more uptake of Evans Blue; $p < 0.05$), whereas MeJ only affected cell viability at level 1 of MCD (16.5 % less uptake; $p < 0.05$). These results are in accordance with the effects observed on biomass accumulation.

Since specific AQ production (expressed μmol gDW^{-1}; see Fig. 3c) was significantly affected by interaction of

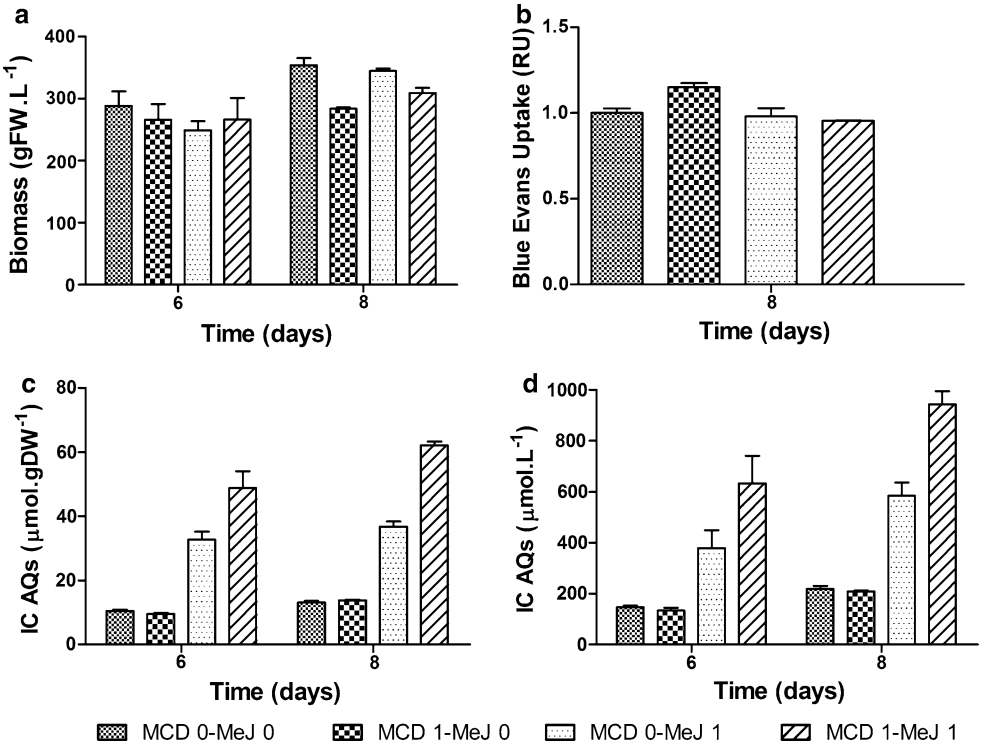

Fig. 3 Results for cell suspension cultures of *R. tinctorum* subjected to the different treatments according to the full factorial design (2^2) with MCD and MeJ. **a** Biomass accumulation, **b** Cell viability; **c** Specific AQ production (expressed as µmol gDW^{-1}); **d** Volumetric AQ production (expressed as µmol L^{-1}). Data correspond to mean values of 3 replicates and *error bars* indicate SD

MCD and MeJ at both 6 ($p = 0.0127$) and 8 days of culture ($p = 0.0001$), simple effects of each elicitor were analyzed. Whereas at 6 days of culture AQ accumulation was not affected by MCD when no MeJ was added (level 0), it showed an increase (48.9 %; $p < 0.05$) in response to MCD when MeJ was present (level 1). On the other hand, MeJ had a profound effect on AQ production at level 0 of MCD (212.4 %; $p < 0.05$), and even a more intense effect at level 1 of MCD (414.1 %; $p < 0.05$) at 6 days of culture. These results are shown in Table 8. The same behavior was observed at 8 days of culture (Table 9). MCD significantly increased AQ content only when at level 1 of MeJ (69.2 %; $p < 0.05$), and MeJ had a deep impact on AQ production at level 0 of MCD (179.2 %; $p < 0.05$), and even greater when MCD was added (349.7 %; $p < 0.05$). Interaction plots are included in a supplemental archive (Figure S5).

MeJ and MCD had a similar effect on volumetric AQ production (expressed as µmol L^{-1}) to that observed for specific production (Fig. 3d). Interaction was significant at both 6 and 8 days of culture ($p = 0.0073$ and $p = 0.0001$, respectively), and the addition of MCD had no effect at level 0 of MeJ. In the presence of MeJ (level 1), MCD increased AQ content at 6 and 8 days of culture (67.2 and a 61.6 %, respectively; $p < 0.05$). On the other hand, the addition of MeJ had a positive effect on AQ accumulation at both days of culture, at level 0 of MCD (155.1 and 167.3 % at 6 and 8 days of culture, respectively; $p < 0.05$), and even more at level 1 of MCD (371.1 and 352.5 % at 6 and 8 days of culture, respectively; $p < 0.05$). These analyses are shown in Tables 10 and 11. Interaction plots are included in a supplemental archive (Figure S6).

Regarding extracellular AQ accumulation, although interaction between both compounds (MeJ and MCD) was significant at both days of culture, the released AQs accounted for a small proportion of total AQs (~5–6 %; data not shown).

Table 8 Simple effects of MCD and MeJ on intracellular AQ production (expressed as µmol gDW^{-1}) in *R. tinctorum* cell cultures after 6 days of culture, analyzed by Tukey's test (with significant MCD–MeJ interaction by two-way ANOVA)

	MCD 0		MCD 1	
	AQs (µmol gDW^{-1})	Result*	AQs (µmol gDW^{-1})	Result
MeJ 0	10.50	A	9.50	A
MeJ 1	32.80	B	48.83	C

* Different letters represent significant differences among medias by Tukey's test ($p < 0.05$)

Table 9 Simple effects of MCD and MeJ on intracellular AQ production (expressed as μmol gDW^{-1}) in *R. tinctorum* cell cultures after 8 days of culture, analyzed by Tukey's test (with significant MCD–MeJ interaction by two-way ANOVA)

	MCD 0		MCD 1	
	AQs (μmol gDW^{-1})	Result*	AQs (μmol gDW^{-1})	Result
MeJ 0	13.17	A	13.83	A
MeJ 1	36.77	B	62.20	C

* Different letters represent significant differences among medias by Tukey's test ($p < 0.05$)

Table 10 Simple effects of MCD and MeJ on intracellular AQ production (expressed as μmol L^{-1}) in *R. tinctorum* cell cultures after 6 days of culture, analyzed by Tukey's test (with significant MCD–MeJ interaction by two-way ANOVA)

	MCD 0		MCD 1	
	AQs (μmol L^{-1})	Result*	AQs (μmol L^{-1})	Result
MeJ 0	148.3	A	134.3	A
MeJ 1	378.3	B	632.7	C

* Different letters represent significant differences among medias by Tukey's test ($p < 0.05$)

Table 11 Simple effects of MCD and MeJ on intracellular AQ production (expressed as μmol L^{-1}) in *R. tinctorum* cell cultures after 8 days of culture, analyzed by Tukey's test (with significant MCD–MeJ interaction by two-way ANOVA)

	MCD 0		MCD 1	
	AQs (μmol L^{-1})	Result*	AQs (μmol L^{-1})	Result
MeJ 0	219.0	A	208.7	A
MeJ 1	585.3	B	944.3	C

* Different letters represent significant differences among medias by Tukey's test ($p < 0.05$)

These results are in accordance with the results observed when HPCD was used. Although both CD had a synergistic effect with MeJ on AQ accumulation in *R. tinctorum* suspension cultures, the response was different in time and intensity. While AQ accumulation was similar between MCD-MeJ and HPCD-MeJ treatments at 6 days of culture (2.40 and 2.38 μmol gDW^{-1}, respectively), it decreased at 8 days of culture in cells treated with HPCD–MeJ (1.98 μmol gDW^{-1}) but it showed an increase in cells treated with MCD-MeJ (3.05 μmol gDW^{-1}). Therefore, it seems that MCD in combination with MeJ provoked a more intense response at longer times (8 days of culture), whereas HPCD combined with MeJ produced a more rapid but mild effect. However, this hypothesis should be confirmed with long-term studies.

Since the more intense effects of CD-MeJ combination were observed on intracellular AQ accumulation and not on AQ release to the culture media, it is clear that the effect of MCD and HPCD was not to enhance stability and/or solubility of these secondary metabolites in the culture media. Recent works showed that CDs induce signaling cascades, including an increase in cytoplasmic calcium and reactive oxygen species (Almagro et al. 2012; Belchi-Navarro et al. 2013). Other authors have reported that combination of MeJ and CDs resulted in changes in the extracellular proteome of different species, especially in proteins related to defense responses and secondary metabolite transport (Briceño et al. 2012; Corchete and Bru 2013; Martinez-Esteso et al. 2009, 2011), and also in the expression of genes related to secondary metabolite biosynthesis (Almagro et al. 2014; Lijavetzky et al. 2008). It is clear that, in some plant species, CD act as elicitors themselves that are able to trigger secondary metabolite accumulation. In fact, it was found that the structure of CDs resembles that of some oligosaccharides released from plant cell walls after a fungal infection (Bru et al. 2006).

Our results are partially in accordance with this evidence, since in our experiments both CDs (MCD and HPCD) synergistically enhanced AQ content in combination with MeJ but when added alone, CDs were unable to trigger AQ accumulation. Recently, it was demonstrated that MeJ forms inclusion complexes with CDs with a 1:1 stoichiometry (López-Nicolás et al. 2013). In this way, the encapsulation within the CD structure affects MeJ solubility and stability, and therefore could have a deep impact on overall elicitation process. Another kind of studies is needed in order to properly stablish the mechanism of AQ accumulation in response to the combination of MeJ and CDs.

Conclusions

AQ production have been achieved by in vitro culture of different species of the Rubiaceae, and several strategies have been employed to increase their content (Bulgakov et al. 2002; Busto et al. 2013; Chong et al. 2005;

Doernenburg and Knorr 1994; Komaraiah et al. 2005; Orbán et al. 2008; Perassolo et al. 2007, 2011b; Quevedo et al. 2012; Vasconsuelo et al. 2003).

In this work, we analyzed the effects of the combination of MeJ, a well-known elicitor, and two CD (HPCD and MCD) on cell cultures of Rubiaceae. These two CDs have been used before alone or in combination with MeJ to synergistically enhanced secondary metabolite production and release to the culture medium (Bru et al. 2006; Sabater-Jara and Pedreño 2013; Sabater-Jara et al. 2014). The experiments were designed according to a full factorial design of two factors (MeJ and a CD) in two levels (0 and 0.1 mM for MeJ, and 0 and 20 mM of either MCD or HPCD) and results were analyzed by two-way ANOVA, in order to detect significant interaction between factors.

We found that the combination between HPCD and MeJ had a negative effect on growth and viability in cell cultures of *M. citrifolia*, which masked the synergistic effect on AQ accumulation (it was only evident when AQ content was expressed as μmol gDW^{-1}). On the other hand, the same treatment had a synergistic effect on AQ accumulation in cell cultures of *R. tinctorum*, whereas no significant effects were observed on growth. It is worth mentioning that even the fact that *M. citrifolia* has a higher AQ content in basal conditions, the strategy assayed in this work (combination of MeJ and HPCD) was more efficient in *R. tinctorum*. This can be explained by the fact that susceptibility to elicitors might be different (Vasconsuelo and Boland 2007), even in species belonging to the same family.

Combination of MeJ with another CD (MCD) resulted in a more intense synergistic effect on AQ accumulation than that observed when MeJ and HPCD were added, revealing a different time-response in the elicitation process.

The synergistic effect described in this work was previously reported by other authors in *V. vinifera*, *S. tuberosum*, *C. annum, C. roseus*, *A. annua* and *T. media* (Almagro et al. 2014; Belchi-Navarro et al. 2012; Durante et al. 2011; Komaraiah et al. 2003; Lijavetzky et al. 2008; Martinez-Esteso et al. 2011; Sabater-Jara et al. 2010, 2014). However, the combination of CD and MeJ failed to induce a drastic AQ release to the culture media, an effect that was reported by other authors (Almagro et al. 2014; Belchi-Navarro et al. 2012; Corchete and Bru 2013; Durante et al. 2011; Komaraiah et al. 2003; Lijavetzky et al. 2008; Martinez-Esteso et al. 2011; Sabater-Jara et al. 2010, 2014).

Nevertheless, our results show that combination of strategies (using a CD and MeJ) was successful to increase secondary metabolite accumulation in suspension cultures. Moreover, the two-factorial design and the analysis by two-way ANOVA proved to be a useful tool for detecting interaction between factors. To our knowledge, this is the first report of synergistic effect of MeJ and CD on AQ accumulation in plant in vitro cultures.

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Authors' contribution MP performed the experimental work and wrote the article. MES collaborated in the experimental work. AMG and JRT supervised the experimental work and the writing.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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