Enhanced extracellular production of trans-*resveratrol in* Vitis vinifera *suspension cultured cells by using cyclodextrins and methyljasmonate*

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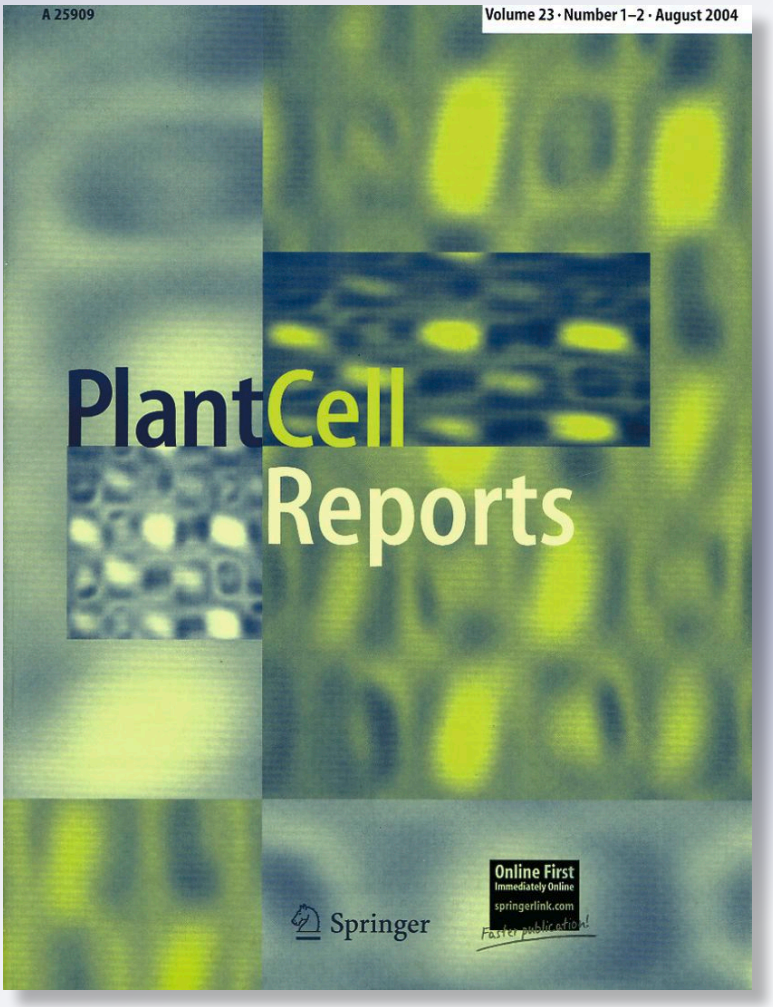

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ORIGINAL PAPER

Enhanced extracellular production of *trans*-resveratrol in *Vitis vinifera* suspension cultured cells by using cyclodextrins and methyljasmonate

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Abstract In this work, the effect of different inducing factors on *trans*-resveratrol extracellular production in Monastrell grapevine suspension cultured cells is evaluated. A detailed analysis provides the optimal concentrations of cyclodextrins, methyljasmonate and UV irradiation dosage, optimal cell density, elicitation time and sucrose content in the culture media. The results indicate that *trans*-resveratrol production decreases as the initial cell density increases for a constant elicitor concentration in Monastrell suspension cultured cells treated with cyclodextrins individually or in combination with methyljasmonate; the decrease observed in cell cultures elicited with cyclodextrins alone is far more drastic than those observed in the combined treatment. *trans*-Resveratrol extracellular production observed by the joint use of cyclodextrins and methyljasmonate (1,447.8 ± 60.4 μmol *trans*-resveratrol g^{-1} dry weight) is lower when these chemical compounds are combined with UV light short exposure (669.9 ± 45.2 μmol *trans*-resveratrol g^{-1} dry weight). Likewise, *trans*-resveratrol production is dependent on levels of sucrose in the elicitation medium with the maximal levels observed with 20 g l^{-1} sucrose and the joint action of cyclodextrins and 100 μM methyljasmonate. The sucrose concentration did not seem to limit the process although it affects significantly the specific productivity since the lowest sucrose concentration is 10 g l^{-1}, the highest productivity is reached (100.7 ± 5.8 μmol *trans*-resveratrol g^{-1} dry weight g^{-1} sucrose) using cyclodextrins and 25 μM methyljasmonate.

Keywords Cyclodextrin · Elicitation · Methyljasmonate · Resveratrol · UV light · *Vitis vinifera*

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Introduction

Vitis vinifera produces stilbenes, which are a small group of compounds characterized by a 1,2-diphenylethylene backbone, derived from the phenylpropanoid pathway. Most plant stilbenes have phytoalexin activity and are derivatives of the monomeric unit *trans*-resveratrol (*t*-R, 3,5,4′-trihydroxystilbene) although other structures are found in other plant families. The formation of stilbenes is considered to be a part of the general defense mechanism since they display strong antifungal and antimicrobial activities (Jeandet et al. 2002; Pezet et al. 2004; Morales et al. 1998). In fact, *t*-R is found in both grapevine tissue and berries, and in cell cultures as a result of both abiotic

and biotic stress (Langcake and Pryce 1977a, b; Pezet et al. 2004; Cantos et al. 2003; Bru et al. 2006). Since *t*-R was postulated to be involved in the health benefits associated with a moderate consumption of red wine (Siemann and Creasy 1992), it is one of the most extensively studied natural products. Hundreds of studies have reported the beneficial effects of *t*-R on neurological (Okawara et al. 2007) and cardiovascular systems (Bradamante et al. 2004). One of the most striking biological activities of *t*-R has been its anticancer activity preventing carcinogenesis in the three stages of tumour development (Pervaiz 2003). More data provide interesting insights into the effect of this compound on the lifespan of different organisms, suggesting that *t*-R could be regarded as a potential anti-aging agent in treating age-related human diseases (De la Lastra and Villegas 2005). In addition, effects described in mice subjected to a high-calorie diet (Baur et al. 2006) point to new approaches for treating not only age-related diseases but also obesity-related disorders (Kaeberlein and Rabinovitch 2006). That is why new strategies based on the use of *V. vinifera* cell cultures have been used to increase *t*-R production (Donnez et al. 2009; Kiselev 2011). One of these strategies includes the use of biotic or abiotic elicitors such as UV light irradiation (Langcake and Pryce 1977b; Keskin and Kunter 2008, 2010), *β*-cyclodextrins (CDs) (Bru and Pedreño 2003; Bru et al. 2006) and methyljasmonate (MJ) (Belhadj et al. 2008; Tassoni et al. 2005; Donnez et al. 2011). Moreover, stilbene and flavonoid production depends on culture conditions such as sugar content (Larronde et al. 1998; Belhadj et al. 2008) and cell density (Akalezi et al. 1999).

The effect of UV irradiation on stilbene content in grapevine suspension cultured cells (SCC) is not known and most of the research related with UV light has led to enhancing the stilbene content of grape berries (Adrian et al. 2000; Cantos et al. 2003), leaves (Langcake and Pryce 1977b; Pezet et al. 2003) and callus tissue (Keskin and Kunter 2008, 2010).

Jasmonic acid and its more active derivative MJ are signal molecules that act as key compounds of the signal transduction pathway involved in the induction of the biosynthesis of secondary metabolites which takes part in plant defence reactions (Gundlach et al. 1992; Creelman and Mullet 1997; Staswick 1998; Chung et al. 2003). Thus, the production of secondary metabolites increases when plant cell cultures are elicited with jasmonates (Gundlach et al. 1992; Blechert et al. 1995; Zhao et al. 2005; Vasconsuelo and Boland 2007). In fact, the production of stilbenes in *V. vinifera* SCC increases by exogenous MJ application (Belhadj et al. 2008; Tassoni et al. 2005).

CDs are cyclic oligosaccharides that chemically resemble the alkyl-derived pectic oligosaccharides naturally released from the cell walls during fungal attack (Bru et al. 2006) and they act as true elicitors since they activate some transcription factors in grapevine cells, inducing the production of *t*-R (Zamboni et al. 2009). They have a hydrophilic external surface and hydrophobic central cavity that trap *t*-R, forming inclusion complexes (Morales et al. 1998). In addition, the high levels of *t*-R accumulated in the culture medium have no toxic effect on the cell lines, allowing successful subcultures. In fact, CDs act not only as inducers of *t*-R biosynthesis but also as promoters of adducts that remove *t*-R from the medium, reducing feedback inhibition and *t*-R degradation and allowing its accumulation in high concentrations (Almagro et al. 2011).

The effects of CDs, MJ and a combination of both on *t*-R extracellular production in grapevine SCC have been previously analysed by Lijavetzky et al. (2008). However, there are no data about how grapevine SCC responds to these elicitors in combination with UV light. Moreover, media and culture conditions are other critical parameters which could influence the improvement of *t*-R production in grapevine SCC.

The final goal of this research is to choose the best operating conditions to achieve high *t*-R levels and to increase *t*-R productivity evaluating the effect of different factors on grapevine SCC. For this, a detailed analysis of these factors will provide the optimal concentrations of inducers and UV irradiation dosage, optimal cell density, time elicitation and sucrose content in the culture media.

Materials and methods

Plant materials

Vitis vinifera L. cv Monastrell calli were established in our laboratory in 1990 as described by Calderón et al. (1993). Since then, calli have been maintained at 25°C in the dark in 250 ml flasks containing 100 ml of fresh growth medium (Gamborg B_5) supplemented with Morel vitamins (Morel 1970), 0.25 g l^{-1} casein hydrolysate, 20 g l^{-1} sucrose, 0.2 mg l^{-1} kinetin, 0.1 mg l^{-1} 1-naphthaleneacetic acid and pH adjusted at 6.0. Grapevine calli were subcultured on solid growth medium every month.

Monastrell SCC were initiated by inoculating friable callus pieces in 250 ml Erlenmeyer flasks containing 100 ml of liquid growth medium adjusted to pH 6.0 and, maintained in a rotary shaker (110 rpm) at 25°C in the dark. Monastrell SCC were routinely maintained by periodical subcultures every 14–16 days by diluting with one

volume of growth medium and then distributing into two flasks.

Elicitor treatments

Elicitation experiments were performed in triplicate using 12–14-day-old Monastrell SCC. At this stage of cell development, 20 g of fresh weight (FW) of washed cells was transferred into 250 ml flasks and suspended in 100 ml of fresh growth medium supplemented with either CDs (Wacker Chemie, Spain) or MJ (Duchefa, Spain) or a combination of both during 96 h of incubation at 25°C in the dark in a rotary shaker (110 rpm). Control treatments without elicitors were always run in parallel. In other cases, elicitation was started with MJ at different concentrations in combination with 50 mM CDs and/or under UV light at short and long exposure times in the presence or in the absence of the above chemical elicitors. For this, Monastrell SCC were transferred to opened polypropylene vessels (without vessel covers), maintained in continuous agitation in a laminar flow hood and directly exposed to UV-C light (254 nm, 10 μW cm^{-2}) for 15 or 120 min at an irradiation distance of 15 cm. After irradiation, SCC were transferred into flasks and maintained in continuous agitation for 96 h. The effect of sucrose content on *t*-R production was assessed by transferring cells into fresh medium supplemented with 10, 20 or 30 g l^{-1} sucrose at the moment the elicitation experiments were carried out, in the presence or in the absence of the above chemical elicitors. After elicitation, the cells were separated from the culture medium under a gentle vacuum and the spent medium was used for measuring the *t*-R content.

Analysis of *trans*-resveratrol in the culture medium

Aliquots of the spent medium were diluted with methanol to a final concentration of 80% methanol (v/v). Thus, 20 μl of diluted and filtered (Anopore 0.2 μm) samples were analyzed in a HPLC-DAD (Waters 600E, Waters 996) as described Bru et al. (2006) using a Spherisorb ODS2 C-18 column (250 × 4.6 mm, 5 μm). *t*-R was identified (at 304 nm) and quantified by comparison with authentic *t*-R of >99% purity (Sigma-Aldrich, Spain).

Determination of cell viability

Cell viability was assessed by incubating the cells for 1–2 min in fresh growth medium containing 100 μg ml^{-1} fluorescein diacetate (DAF) (Huang et al. 1986) and observing the fluorescence emission by living cells with a DMRB Leica optical microscope ($\lambda_{exc} = 490$ nm, $\lambda_{emi} = 520$ nm) using a Leica filter I3.

Results and discussion

Cell growth and extracellular production of *trans*-resveratrol is dependent on MJ dose and CDs

The effect of the elicitors on cell growth was checked by determining cell dry weight (DW) (initially in all treatments, 200 g FW l^{-1} which is equivalent to 10 g DW l^{-1}) after 96 h of treatment. As shown in Fig. 1a, the DW of MJ-treated cells as well as that of the cells treated with CDs + MJ decreased (up to 60%) while cell growth remained constant in control cells, in cells treated only with CDs or with the lowest MJ concentration (5 μM). These results show that the joint action of CDs plus MJ as well as MJ treatments leads to a reduction of cell growth. In fact, MJ is associated not only to induce the production of stilbenes but also to modify the pattern of cell growth in grapevine SCC (Krisa et al. 1999). Tassoni et al. (2005) described the undesirable effect of a reduction of cell growth in MJ treated *V. vinifera* cv Barbera cell cultures. Donnez et al. (2011) also described a change of cell colour and an inhibition of cell growth in MJ treated 41B (*V. vinifera* cv Chasselas × *V. berlandieri*) cell cultures. Kiselev et al. (2007) also described the inhibition of cell growth in *V. amurensis* callus cultures as a result of treatment with different elicitors. This effect of MJ on cell growth has been suggested to be caused by the diversion of the metabolic flux (carbon allocation), favouring the activation of the secondary metabolism over the primary metabolism (Larronde et al. 2003; Sivakumar and Paek 2005), although a direct effect of MJ on the repression of cell multiplication and/or growth has been reported by Pauwels et al. (2008). Although cell growth was halted in elicitor-treated cells, cell viability, as assessed by fluorescence microscopy of cells treated with DAF (Fig. 2b), seems to be unaffected in comparison with control cells (Fig. 2a).

Figure 1b shows the effect of different concentrations of MJ on *t*-R productivity in Monastrell SCC during 96 h, in the absence or in the presence of CDs. As shown in Fig. 1b, the maximum level of *t*-R produced and secreted by cells to the medium was reached when SCC were incubated with 50 mM CDs and 100 μM MJ (1,447.8 ± 60.4 μmol *t*-R g^{-1} DW), this level being around 4.3-fold higher than when cells were treated only with CDs (338.6 ± 51.3 μmol *t*-R g^{-1} DW). However, in the presence of MJ alone, very low amounts of *t*-R were detected (16.4 ± 2.0 μmol *t*-R g^{-1} DW). No *t*-R was detected in cells treated with ethanol, the solvent in which MJ is delivered to the culture (data not shown). Krisa et al. (1999) described that the amount of total stilbenes secreted to the culture medium when 25 μM MJ was added in any tested conditions was negligible in both MJ and control cultures of three *V. vinifera* cultivars.

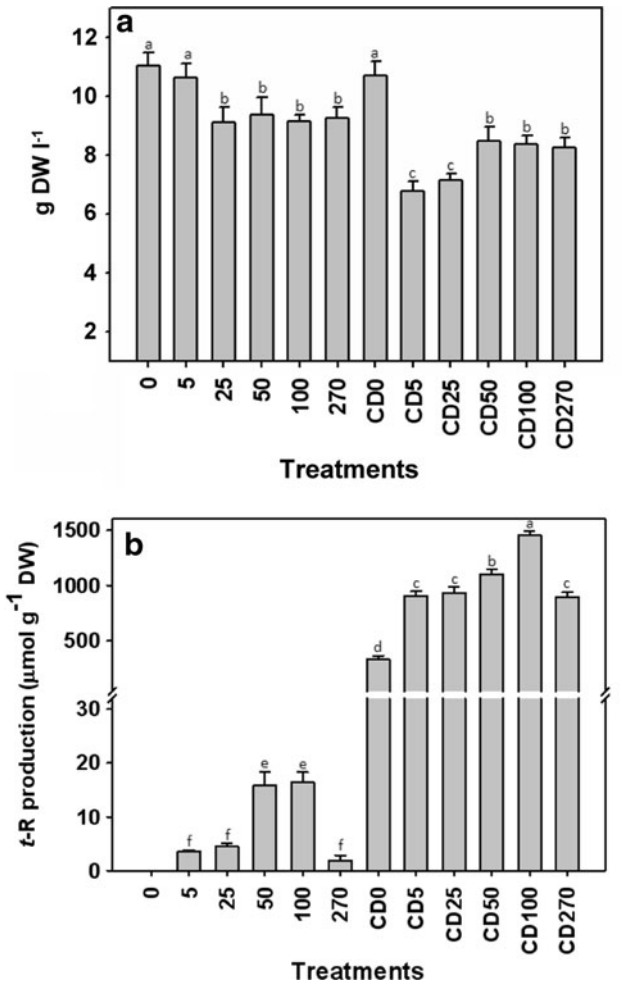

Fig. 1 Effect of different MJ concentrations (5, 25, 50, 100 and 270 µM) on growth **a**, measured as g DW l^{-1}, and *t*-R production **b**, expressed as µmol g^{-1} DW, in Monastrell SCC in the absence or in the presence of 50 mM CDs. The initial cell density in all the treatments was 10 g DW l^{-1}. Results were evaluated 96 h after treatments. Experiments were carried out with 12–14-day-old Monastrell cells and were repeated twice. Data are the mean ± SD of the replicates

In the presence of 25 µM MJ, we detected levels of *t*-R in the culture medium of 4.5 ± 0.6 µmol *t*-R g^{-1} DW in Monastrell SCC (Fig. 1b). However, extracellular production of *t*-R increased 186.48 times (932.5 ± 55.1 µmol *t*-R g^{-1} DW) when 25 µM MJ and 50 mM CDs were jointly used as inducers (Fig. 1b). Moreover, Tassoni et al. (2005) showed that the addition of MJ (10 µM), at day 0, was effective in stimulating endogenous *t*-R accumulation (around 105 nmol g^{-1} DW), as well as promoting its release into the medium (over 35 nmol g^{-1} DW) in *V. vinifera* cv Barbera cell cultures. In our case, the extracellular *t*-R production in the presence of 5 µM MJ was 3.5 ± 0.6 µmol *t*-R g^{-1} DW and in the presence of both elicitors, 905.0 ± 40.0 µmol *t*-R g^{-1} DW. Although not all grapevine cell lines respond in the same way to the presence of MJ and the levels of *t*-R production are dependent on grapevine cultivar, the addition of CDs, separately or in combination with MJ, highly enhanced extracellular production of *t*-R.

Extracellular production of *trans*-resveratrol is dependent on elicitation time course

The time course of *t*-R production in Monastrell SCC treated with CDs and MJ, separately or in combination, was followed by periodically analyzing the *t*-R concentration in the culture medium (Table 1). No *t*-R was detected in control SCC and very low amounts of *t*-R were found in MJ-treated cells (37.4 ± 4.5 mg *t*-R l^{-1} in the best conditions with 100 µM MJ) in contrast with those results obtained by Donnez et al. (2011) using 41B cell cultures elicited for 96 h with 200 µM MJ (150.8 mg l^{-1}). This type of result could be due to the response of grapevine cells to the presence of MJ. As can be observed in Table 1 in both cases (using only CDs or a combination of CDs + MJ), a continuous extracellular production of *t*-R (measured as mg l^{-1}) and *t*-R productivity (mg l^{-1} day^{-1}) was observed. The higher accumulation rate in the combined treatment compared with the CD treatment and the larger accumulation with elicitation time course are the driving forces behind the greater overall production and productivity of *t*-R. Since *t*-R production in the combined treatment was higher than the sum of the individual treatments (due to the low *t*-R production in MJ-treated cells), a synergistic effect between both elicitors is assumed, as described Lijavetzky et al. (2008). Zamboni et al. (2006) analysed the variations on *t*-R levels in response to CDs in four different grape genotypes. Thus, SCC obtained from the crossing between *V. riparia* and *V. berlandieri* and *V. amurensis* cell lines were capable of producing more extracellular production of *t*-R (911.25 and 225.22 mg l^{-1}, respectively) than grapevine SCC of Pinot Noir (0.51 mg l^{-1}) or Merzling (4.31 mg l^{-1}) after 48 h of CD treatment. The results obtained by these authors using highly productive cell lines are in accordance with those obtained from Monastrell SCC (Table 1) since *t*-R productivity in CD-treated cells obtained from the crossing between *V. riparia* and *V. berlandieri* (455.6 mg l^{-1} day^{-1}) was very close to *t*-R productivity in Monastrell SCC (467.9 ± 1.0 mg l^{-1} day^{-1}, Table 1). Differences in *t*-R levels among *Vitis* genotypes could be related with their different levels of defence response. This variability of the response of *Vitis* cultures to elicitation in the presence of CDs under similar conditions demonstrates that it is very important to select genotypes which have the ability to produce the highest levels of *t*-R.

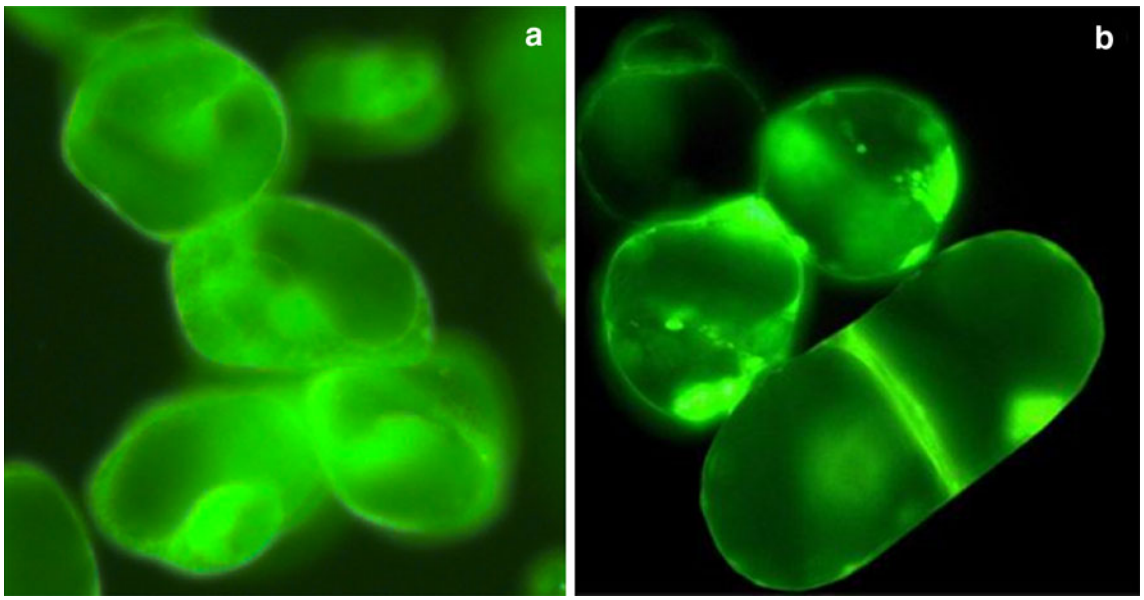

Fig. 2 Cell viability (assessed by DAF as described in "Materials and methods") of Monastrell control **a** and elicited cells **b** 96 h after treatments

Table 1 Effect of elicitation time course on *t*-R production and productivity in Monastrell SCC treated with 50 mM CDs individually and in combination with 100 μM MJ

Time (h)	*t*-R production (mg l^{-1})		*t*-R productivity (mg l^{-1} day^{-1})	
	CDs	CDs + MJ	CDs	CDs + MJ
4	2.1 ± 0.3	9.0 ± 0.1	–	–
24	127.6 ± 0.1	325.2 ± 3.9	127.6 ± 0.1	325.2 ± 3.9
72	499.5 ± 9.9	1,403.9 ± 3.1	166.5 ± 3.3	467.9 ± 1.0
96	539.3 ± 9.7	1,542.7 ± 8.1	134.8 ± 2.4	385.7 ± 2.0
120	522.2 ± 8.1	2,032.9 ± 8.8	104.4 ± 1.6	406.6 ± 1.8
144	646.9 ± 2.1	2,686.4 ± 9.5	107.8 ± 0.4	447.7 ± 1.6
168	737.0 ± 4.6	3,047.6 ± 9.4	105.3 ± 0.7	435.4 ± 1.3
216	557.4 ± 9.9	3,035.8 ± 13.6	61.9 ± 1.1	337.3 ± 1.6
240	594.4 ± 10.6	3,056.0 ± 15.2	59.4 ± 1.1	305.6 ± 1.5
288	628.6 ± 14.6	3,038.9 ± 18.4	52.4 ± 1.2	253.3 ± 1.6

Experiments were repeated twice. Data are the mean ± SD of the replicates

Extracellular production of *trans*-resveratrol is dependent on initial cell density for a constant elicitor concentration

Table 2 shows how extracellular production of *t*-R decreased as the initial cell density increased in Monastrell SCC treated with CDs individually or in combination with MJ. In fact, the maximum levels of *t*-R are reached with a low cell density (5 g DW l^{-1}). These results could be explained by the use of the same quantity of elicitors on different initial cell densities since the cell reaction could rise when the cell quantity is low by increasing the number of receptors in cell membranes. This behaviour is also observed in Gamay SCC since the maximal levels of *t*-R are reached with low cell densities (unpublished results). However, the levels of *t*-R in all the combined treatments (CDs + MJ) are higher than those in the absence of MJ. In fact, when *t*-R values were normalized considering the production at low cell density as 100%, the decrease in *t*-R production was more drastic for the treatment with CDs alone than for the combined treatment. Hence, we decided to work at intermediate cell density (10 g DW l^{-1}), using both CDs and MJ and to explore the possibility of increasing *t*-R production through the addition of a third elicitor, in this case, UV light.

Effect of UV light exposure and both CDs and MJ on extracellular production of *trans*-resveratrol

UV light has been described as a physical elicitor of stilbene biosynthesis in grapevine (Douillet-Breuil et al. 1999; González-Barrio et al. 2006). Hence, the effect of the

Table 2 Effect of different initial cell densities on *t*-R production in Monastrell SCC elicited for 96 h with CDs or in combination with MJ

Initial cell density (g DW l^{-1})	CDs		CDs + MJ	
	t-R production (μmol *t*-R g^{-1} DW)	Normalized production (%)	*t*-R production (μmol *t*-R g^{-1} DW)	Normalized production (%)
5	654.2 ± 36.1	100	1,644.3 ± 36.4	100
10	308.0 ± 50.8	47	1,395.6 ± 50.5	80
20	72.3 ± 5.1	11	968.2 ± 55.8	59

Experiments were repeated twice. Data are the mean ± SD of the replicates. Values of production were normalized considering the production at low initial cell density as 100%

exposure of grapevine SCC to UV light in the presence of CDs, separately or in combination with MJ, on *t*-R production was studied. As observed in Fig. 3, grapevine SCC elicited with CDs and exposed for short times to UV light showed a similar increase in *t*-R levels, as seen in UV-unexposed CD-treated cells, while prolonged exposure to UV light caused a drastic reduction in *t*-R accumulation. Likewise, in UV exposed conditions, neither cell browning nor *cis*-R was observed. Moreover, Monastrell SCC treated with CDs and MJ, followed by short or long exposures to UV light, showed lower *t*-R levels than UV-unexposed cells, so that UV light exposure was clearly detrimental to *t*-R production. In fact, grapevine SCC treated with and without MJ and exposed to long UV light produced a negligible amount of *t*-R and SCC browning (data not shown). This negative effect of UV light on *t*-R production in Monastrell SCC is neither due to the experimental conditions [because of SCC sensitivity to the UV light (Almagro et al. 2011)], nor experimental design since SCC were directly exposed to UV light in opened polypropylene vessels (without vessel covers). Therefore, these results could be explained by the joint action of MJ and UV light since SCC treated with CDs + MJ and their exposition to UV light at any time produced a decrease of *t*-R production in comparison with the non-exposed UV cells.

To our knowledge, there are no reports on extracellular *t*-R production in grapevine SCC elicited with UV light. Likewise, most of the research related to UV light has been directed at enhancing the stilbene content of grape berries (Adrian et al. 2000; Cantos et al. 2003), leaves (Pezet et al. 2003) and callus tissue (Keller et al. 2000; Keskin and Kunter 2008). Keller et al. (2000) found that only actively growing Cabernet-Sauvignon calli irradiated with UV light were capable of producing stilbenes, whereas old calli had lost this ability. Similar results were described by Keskin and Kunter (2008) working with Cabernet-Sauvignon calli cultures irradiated with UV light. They found that the effect of UV light on *t*-R production is dependent on callus age since the highest *t*-R production was found in 12-day-old calli (62.66 ± 0.40 μg *t*-R g^{-1} FW) in comparison with those values obtained in 15-day-old calli (18.12 ± 0.10 μg

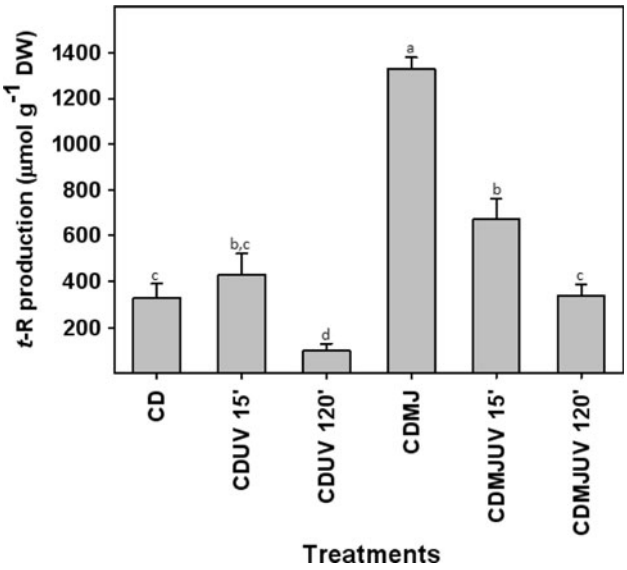

Fig. 3 Effect of the exposure of Monastrell SCC to UV light (15 and 120 min) in the presence of CDs individually or in combination with MJ for 96 h. Values are given as the mean ± SD of the replicates

t-R g^{-1} FW) at the same irradiation time. These responses are similar to that found in ripening grape berries, which gradually lose their potential for synthesizing stilbenes as they approach maturity. Therefore, the low levels of extracellular *t*-R found when Monastrell SCC were exposed to UV light might also be explained taking into consideration that elicitation experiments were performed in 12–14-day-old grapevine SCC that are just entering their stationary phase.

Extracellular production of *trans*-resveratrol is dependent on the level of sucrose in the culture medium

It is well known that an increase of sucrose concentration results in a proportional increase in cell biomass but a similar proportional increase in metabolite production is not always obtained since the increase in carbon source might have a positive or negative effect on metabolite concentration (Zhong and Yoshida 1995). Hence, we have

studied the effect of different sucrose concentrations (10, 20 or 30 g l^{-1}) on extracellular production of *t*-R in Monastrell SCC. For this, filtered fresh cells were transferred into fresh medium that contained different sucrose concentrations, 50 mM CDs and different MJ concentrations (as described in "Materials and methods") at the moment the elicitation experiments were carried out (Fig. 4). As can be observed, the maximal levels of *t*-R production are dependent on both sucrose levels and elicitors added in the culture media. Thus, when grapevine SCC were elicited in a culture medium with 10 g l^{-1} sucrose (white bars), the maximal *t*-R production (1,006.9 ± 58.1 μmol *t*-R g^{-1} DW) was reached using the lowest MJ dose (25 μM), and this production was maintained even when Monastrell SCC are elicited with higher MJ concentrations. The highest *t*-R production using 30 g l^{-1} sucrose was obtained at 50 μM MJ (1,210.8 ± 40.1 μmol *t*-R g^{-1} DW) but the difference was not significant as compared with the previous treatment. Conversely, the maximal levels of *t*-R using 20 g l^{-1} sucrose which are reached at 100 μM MJ (grey bars) (1,447.7 ± 60.4 μmol *t*-R g^{-1} DW) are significantly higher that the maximal levels at any other sucrose concentration tested. In contrast, when no MJ is added as co-elicitor, the lowest *t*-R production values are found in these treatments but no significant differences in *t*-R production are seen at the different sucrose concentrations tested. Belhadj et al. (2008) obtained a *t*-R production of 1.0 μmol *t*-R g^{-1} DW when *V. vinifera* cv. Gamay SCC were elicited with 20 μM MJ in a culture medium with 27.4 g l^{-1} sucrose. Working in similar experimental conditions (25 μM MJ and 30 g l^{-1} sucrose) but in the presence of CDs, the extracellular production in Monastrell SCC was 741.2 ± 19.2 μmol *t*-R g^{-1} DW, i.e., 713-fold higher than those obtained by Belhadj et al. (2008).

The *t*-R productivity per day shows logically the same trend that the overall production; it reaches the highest value of 361.9 ± 15.1 μmol *t*-R g^{-1} DW day^{-1} with 20 g l^{-1} sucrose and 100 μM MJ in comparison with 251.7 ± 14.5 μmol *t*-R g^{-1} DW day^{-1} obtained in 10 g l^{-1} sucrose and 25 μM MJ. However, if we consider the specific *t*-R productivity in relation to sucrose consumption, with 10 g l^{-1} sucrose and 25 μM MJ, the results were 100.7 ± 5.8 μmol *t*-R g^{-1} DW g^{-1} sucrose, while with 20 g l^{-1} sucrose and 100 μM MJ the value was 72.4 ± 3.0 μmol *t*-R g^{-1} DW g^{-1} sucrose. Therefore, the specific productivity was affected by the carbon source concentration which is a parameter relevant to the economy of the process. In conclusion, to increase *t*-R productivity using this highly productive cell line, the best operating conditions for the subsequent scale-up experiments involve long elicitation times of Monastrell SCC (up to 168 h) with a combination of CDs and MJ without any UV light exposure since this decreases *t*-R productivity in all the cases. The CD concentration is fixed at 50 mM since a previous work showed that the use of this concentration led to the highest *t*-R production in this and other grapevine cell lines (Bru et al. 2006). The optimal MJ concentration depended on the sucrose concentration in the culture medium in which elicitation was carried out. The sucrose concentration did not seem to limit the process although it significantly affected the specific productivity. The production of *t*-R increased as inoculum cell density decreased for a constant elicitor concentration although the decrease observed in Monastrell SCC elicited with CDs alone was far more drastic than those observed in the combined treatment with CDs and MJ.

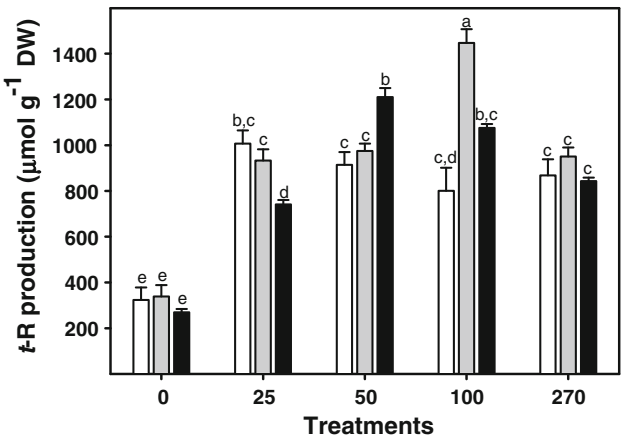

Fig. 4 Effect of sucrose concentration in the elicitation medium on *t*-R production in elicited Monastrell SCC for 96 h. Data are the mean ± SD of the replicates. *White*, *grey* and *black bars* correspond to 10, 20 and 30 g l^{-1} sucrose, respectively

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