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

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Abbreviations

4-coumarate-CoA ligase: 4CL; cinnamate 4-hydroxylase: C4H; coronatine: Cor; dry weight: DW; fresh weight: FW; isoleucine-conjugated form of jasmonic acid: JA-Ile; phenylalanine ammonia lyase: PAL; stilbene synthase: STS; suspension cultured cells: SCC; *trans*-resveratrol: *trans*-R; β -cyclodextrins: CD

Abstract

In the present work the effect of cyclodextrin and coronatine on both *trans*-resveratrol production and the expression of stilbene biosynthetic genes in *Vitis vinifera* L. cv Monastrell suspension cultured cells were evaluated. The results showed the maximum level of *trans*-resveratrol produced by cells and secreted to the culture medium with 50 mM cyclodextrins and 1 μ M coronatine. Since the levels of *trans*-resveratrol produced in the combined treatment were higher than the sum of the individual treatments, a synergistic effect between both elicitors was assumed. In addition, all the analyzed genes were induced by cyclodextrins and/or coronatine. The expression of the *phenylalanine ammonia lyase* and *stilbene synthase* genes was greatly enhanced by coronatine although an increase in the amount of *trans*-resveratrol in the spent medium was not detected. Therefore, despite the fact that *trans*-resveratrol production is related with the expression of genes involved in the biosynthetic process, other factors may be involved, such as post-transcriptional and post-traductional regulation. The expression maximal levels of *cinnamate 4-hydroxylase* and *4-coumarate-CoA ligase* genes were found with cyclodextrins alone or in combination with coronatine suggesting that the activity of these enzymes could be not only important for the formation of intermediates of *trans*-R biosynthesis but also for those intermediates involved in the biosynthesis of lignins and/or flavonoids.

Keywords

Coronatine; cyclodextrins; gene expression; grapevine suspension-cultured cells; *trans*-resveratrol

1. Introduction

Vitis vinifera produces stilbenes, which are a small group of compounds characterized by a 1,2-diphenylethylene backbone. Most plant stilbenes are derivatives of the monomeric unit *trans*-resveratrol (*trans*-R, 3,5,4'-trihydroxystilbene, Jeandet et al., 2014). The formation of stilbenes is considered to be a part of the general defense mechanism since they display strong antifungal and antimicrobial activities (Pezet., 2004; Bru et al., 2006; Adrian et al., 2012). In fact, *trans*-R is produced in both grapevine vegetative tissues and berries as well as in suspension cultured cells (SCC) in response to abiotic and biotic stress (Cantos et al., 2003; Wang et al., 2010). Since *trans*-R was also 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 natural products studied. In fact, hundreds of studies have reported the

beneficial effects of *trans*-R on neurological system (Okawara et al., 2007), cardiovascular diseases (Bradamante et al., 2004), preventing carcinogenesis (Vang et al., 2011; Fernández-Pérez et al., 2012) and as an antiaging agent in the treatment of age-related human diseases (De la Lastra and Villegas 2005).

On the other hand, *trans*-R is biosynthesized from phenylalanine which is a key intermediate linking the primary metabolism and the secondary metabolism. Thus, the first step in the stilbene biosynthesis pathway consists in the transformation of phenylalanine into cinnamic acid in a reaction catalyzed by the enzyme phenylalanine ammonia lyase (PAL). The consecutive action of cinnamate 4-hydroxylase (C4H) and 4-coumarate-CoA ligase (4CL) transforms cinnamic acid into 4-coumaroyl-CoA, which is the common precursor of most of the phenolic compounds found in plants: lignins, flavonoids and stilbenoids. Then, one molecule of 4-coumaroyl-CoA is condensed consecutively with three malonyl-CoA units to produce either *trans*-R, through the action of stilbene synthase (STS), or naringenin chalcone by the action of chalcone synthase (Almagro et al., 2013 and references therein).

Stilbene biosynthesis is induced in response to a wide range of biotic and abiotic elicitors, which in turn, activate the expression of genes encoding stilbene biosynthesis pathway (Ferri et al., 2009; Tassoni et al., 2005). Stilbene biosynthesis is triggered by signalling molecules such as methyl jasmonate (Kiselev 2011). In this way, upon perception of jasmonate signals, the plant cell activates several defense mechanisms, reflected in a massive reprogramming of gene expression which leads to both the activation of stilbene biosynthesis and the expression of pathogenesis related-proteins (Tassoni et al., 2005; Donnez et al., 2011; Kiselev 2011; Belchí-Navarro et al., 2012). Moreover, coronatine (Cor) is a phytotoxin produced by *Pseudomonas syringae* (Bender et al., 1999), that acts as a mimic molecule of the isoleucine-conjugated form of jasmonic acid (JA-Ile) (Katsir et al., 2008). This compound is important for its potential to act as both plant growth regulator and elicitor of plant secondary metabolism (Onrubia et al., 2013).

As Cor resembles the JA-Ile their mode of action could be similar. The actions of Cor include the induction of jasmonic acid biosynthesis, inhibition of root elongation, hypertrophy, chlorosis, ethylene emission, secondary metabolite production, among others (Tamogami and Kodama 2000; Yao et al., 2002; reviewed in Uppalapati et al., 2005). However, although the action of jasmonic acid or methyl jasmonate on secondary metabolite biosynthesis has been widely studied (Tassoni et al., 2005; Lee-Parson et al., 2004), there are relatively few reports on the effect of Cor on secondary metabolite production. In this sense, Onrubia et al. (2013) observed that Cor enhanced both taxane production and expression of

genes encoding enzymes involved in the biosynthesis of taxanes in *Taxus media* SCC. In addition, Cor also increased the accumulation of benzo[c]phenanthridine alkaloid and glyceollins in *Eschscholzia californica* and *Glycine max* L SCC, respectively (Haider et al., 2000; Fliegmann et al., 2003).

On the other hand, β -cyclodextrins (CD) are cyclic oligosaccharides that chemically resemble to the alkyl-derived pectic oligosaccharides naturally released from the cell walls during fungal attack (Bru et al., 2006). They act as true elicitors since they provoke stilbene accumulation and induce the accumulation of new gene products like peroxidases, β -1,3-glucanases and chitinases (Morales et al., 1998; Martínez-Esteso et al., 2009). They have a hydrophilic external surface and hydrophobic central cavity that trap *trans*-R, forming inclusion complexes (Morales et al., 1998). In addition, the high levels of *trans*-R which is secreted and accumulated in the culture medium have no toxic effect on the cell lines, allowing successful subcultures. In fact, CD act not only as inducers of *trans*-R biosynthesis but also as promoters of adducts that remove *trans*-R from the medium, reducing feedback inhibition and *trans*-R degradation and allowing its accumulation in high concentrations (Almagro et al., 2011). Interestingly, Lijavetzky et al. (2008) demonstrated that the combined use of CD and methyl jasmonate enhanced the production of *trans*-R, which was strongly associated to an increased expression of *STS*, *PAL*, *C4H*, *4CL* genes in grapevine SCC. However, the effect of Cor in combination with others elicitors has never been studied on both *trans*-R production and phenylpropanoid pathway gene expression.

Taking into account all of the above, in the present study we have analyzed the influence of CD and/or Cor on the expression profiles of several biosynthetic pathway genes associated with the production of *trans*-R in *V. vinifera* Monastrell SCC.

2. Material and Methods

2.1 Plant material

Vitis vinifera L. cv Monastrell calli were established in our laboratory in 1990 as described by Calderon 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 B₅) 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. *V. vinifera* SCC were initiated by inoculating friable callus pieces (20 g fresh weight (FW)) 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. *V. vinifera* SCC were routinely maintained by periodical subcultures every 15 days.

2.2 Elicitor treatments

Elicitation experiments were performed in triplicate using 15 day old *V. vinifera* SCC. At this stage of cell development, 4 g of FW of washed cells were transferred into 100 mL flasks, suspended in 20 ml of fresh growth medium supplemented with either 50 mM CD (Wacker Chemie, Spain) or 0.5, 1 or 2 μ M Cor (Sigma, Spain) alone or in combination, and maintained at 25 °C in the dark in a rotary shaker (110 rpm). Control treatments without elicitors were always run in parallel. After elicitation, cells were separated from the culture medium by filtration, rapidly washed with cold distilled water, weighted and frozen at -80 °C until use. The elicited culture medium was used for *trans*-R quantification.

2.3 Quantification of *trans*-resveratrol in both culture medium and cells

For this, 20 μ L of the spent medium were analyzed in a HPLC-DAD (Waters 600E, Waters 996) as described by Belchí-Navarro et al. (2012). In addition, 50 mg of freeze-dried cells were extracted overnight in 4 mL methanol at 4°C. The cell extract was diluted with water to a final concentration of 80% (v/v) methanol. Then, 20 μ L of the diluted extract was filtered (Anopore 0.2 μ m) and analyzed in a HPLC-DAD (Waters 600E, Waters 996) as described by Bru et al. (2006) using a Spherisorb ODS2 C-18 column (250 x 4.6 mm, 5 μ m). *trans*-R was identified at 306 nm and quantified by comparison with authentic standard of >99% purity (Sigma-Aldrich, Spain).

2.4 Gene expression analyses

RNA isolation, cDNA synthesis and real-time quantitative PCR

Total RNA was extracted from frozen cells (0.5 g FW) by means of the TRIZOL reagent (Invitrogen) following the manufacture's recommendations. RNA quality was verified with a Bioanalyzer instrument (Bioanalyzer 2100, Agilent Technologies). RNA concentration was determined optically with a Helios Gamma spectrophotometer (Thermo Scientific). Only RNA preparations with A260/A280 ratios of 1.8–2.0 and A260/A230 ratios of 2.0 were used for subsequent analysis. RNA integrity was verified by 2 % agarose gel electrophoresis RNAase free followed by GelRed staining. First strand cDNA was synthesized from 0.2 μ g of

total RNA using the iScript™ Select cDNA Synthesis Kit (Bio-Rad) with the Oligo (Dt) primers mix.

The qRT-PCR procedures were performed using the primers designed by Lijavetzky et al. (2008). The cDNA samples were analyzed with an iCycler (Bio-Rad) apparatus using SYBR Green PCR Core Reagents (Life Technologies) and the results were analyzed with the manufacturer's software (iCycler Optical System Software, v. 3.0.6; BioRad). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C. For each primer pair, its reaction efficiency was assessed from a standard curve generated from a serial dilution of pooled cDNA. For each gene, the expression values were normalized with respect to grapevine EF α gene used as reference control as described by Lijavetzky et al. (2008) and Almagro et al. (2014). In addition, the relative expression levels of all other samples were then expressed in relation to the expression of control cells (untreated cells), and the resulting Ct values were processed according to Pfaffl (2001). Relative expression values of the different genes were analyzed at 0, 8, and 24 h after elicitation treatments. In all cases, each PCR was performed with at least three independent samples.

2.5 Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test in order to examine the significance of the observed differences using the SPSS package (SPSS Inc., Chicago, USA) version 19.0, and *P* values <0.05 were considered as statistically significant.

3. Results and Discussion

3.1 Effect of different concentrations of coronatine in the presence of cyclodextrins on *trans*-resveratrol production

In order to check what was the concentration of Cor effective in enhancing the production of *trans*-R, we analysed the effect of different Cor concentrations on extracellular *trans*-R accumulation in SCC of *V. vinifera* elicited for 96 h in the presence of 50 mM CD (Fig. 1). The amount of *trans*-R induced by CD alone was 201 ± 56 mg.L⁻¹ while the maximum level of *trans*-R produced by cells and secreted to the culture medium was reached when SCC were incubated with 50 mM CD and 1 μ M Cor (684 ± 45 mg.L⁻¹). Moreover, in the presence of 50 mM CD with 0.5 μ M Cor or 2 μ M Cor the levels of *trans*-R were lower (543 ± 35 and 421 ± 67

mg.L⁻¹, respectively). In agreement with our results, Weiler et al. (1994) and Onrubia et al. (2013) reported that 1 μM Cor induced high levels of indole alkaloids and taxanes in *Rauwolfia serpentine* and *Taxus media* SCC, respectively. In addition, we did not detect any increase in extracellular production of *trans*-R when SCC were only elicited with Cor (data not shown).

3.2 The extracellular and intracellular production of *trans*-resveratrol is dependent on elicitation time course

In order to compare the effects of the different treatments on the production of *trans*-R in the SCC, the level of this compound was analyzed both in cells and extracellular medium at 0, 6, 24, 72, 96 and 168 h. As shown in Fig. 2, no *trans*-R was detected in control SCC and a insignificant amount of *trans*-R was detected in the extracellular medium when SCC were only elicited with Cor (not observable in the Fig. 2). In contrast, the level of extracellular *trans*-R increased up to 72 h in CD-treated cells (250,21±61,12 mg.L⁻¹), and when cells were simultaneously elicited with CD and Cor, the accumulation of *trans*-R in the culture medium increased more than twice (942,96±120,03 mg.L⁻¹) (Fig. 2). Since the levels of *trans*-R produced in the combined treatment was higher than the sum of the individual treatments (due to the negligible *trans*-R production in Cor-treated cells), a synergistic effect between both elicitors is assumed. Similarly, a synergistic effect on *trans*-R production was observed when *V. vinifera* SCC were elicited with CD and methyl jasmonate (Belchí-Navarro et al., 2012) reaching levels about 1600 mg.L⁻¹ after 96 h of treatments. Therefore, both Cor and methyl jasmonate produced a synergistic effect on *trans*-R biosynthesis in the presence of CD indicating that Cor was able to mimic the biologically active form of jasmonic acid, methyl jasmonate. A synergistic effect of CD and Cor on taxane production was also observed by Ramirez-Estrada et al. (2015) in *Taxus* SCC. In fact, the combined treatment provoked an increase over 40- and 17.6-fold higher than in the control cells in *Taxus media* and *T. globosa* SCC, respectively.

Similarly, the highest *trans*-R levels detected in cells were reached in the presence of CD and Cor after 168 h of treatment (79 ± 11 μg. g dry weight⁻¹ (DW) that means 0.79 mg.L⁻¹) (Fig. 3) although it was nearly three order of magnitude lower than the final concentration obtained in the spent medium in the same conditions (942,96±120,03 mg.L⁻¹, that is 95 mg.g DW⁻¹). In addition, the intracellular concentration of *trans*-R in the presence of Cor were almost similar

in all time studied reaching maximal levels at 24 h of treatment (around $10 \mu\text{g.g DW}^{-1}$ that means 0.1 mg.L^{-1}) (Fig. 3). In agreement to our results, an increase in intracellular production of *trans*-R (1.6 mg.g DW^{-1}) was detected in *V. vinifera* cv Negramaro elicited with a ten times higher concentration of Cor ($10 \mu\text{M}$) for 144 h (Taurino et al., 2015). Therefore, Cor provoked an induction of stilbene biosynthetic pathway leading to intracellular accumulation of *trans*-R in *V. vinifera* SCC.

3.3 Cell growth is dependent on the presence of elicitors

The effect of the elicitors on cell growth was checked by determining the final cell DW (expressed as g DW.L^{-1}) after 168 h of treatment. As shown in Fig. 4, the final DW of Cor-treated cells ($26,50 \pm 0,28 \text{ g DW.L}^{-1}$) as well as that of the cells treated with CD separately ($25,00 \pm 0,27 \text{ g DW.L}^{-1}$) or in combination with Cor ($25,00 \pm 0,52 \text{ g DW.L}^{-1}$) decreased (around 30%) based on the weight reached in control cells ($35,12 \pm 0,70 \text{ g DW.L}^{-1}$). Our results agree with those found by Taurino et al. (2015) who observed that the addition of $10 \mu\text{M}$ Cor to *V. vinifera* cv Negramaro SCC provoked a decrease on cell growth, showing a reduction of 40% after 144 of treatments. Similarly, Onrubia et al. (2013) showed that the capacity for biomass formation of Cor-elicited cells was slightly lower than control cells in *Taxus media*. Because of Cor has a biological activity similar to methyl jasmonate, we hypothesized that Cor could act in the same way that methyl jasmonate, diminishing cell growth and so, DW in grapevine SCC. In fact, Belchí-Navarro et al. (2012) showed that the addition of methyl jasmonate leads to a reduction of cell growth in *V. vinifera* SCC. Donnez et al. (2011) also described an inhibition of cell growth of *V. vinifera* cv Chasselas and *V. berlandieri* SCC in the presence of methyl jasmonate. This effect of methyl jasmonate on cell growth could be due to the deviation of the metabolic flux (carbon allocation), favouring the activation of the secondary metabolism over the primary metabolism (Sivakumar and Paek 2005), although a direct effect of methyl jasmonate on the repression of cell growth has been reported by Pauwels et al. (2008).

3.4 Time course of *trans*-resveratrol gene expression in grapevine suspension cultured cells elicited with cyclodextrins and/or coronatine

In order to analyze the relationship between the *trans*-R accumulation, and *trans*-R biosynthetic pathway gene expression under elicitation, we performed a real time qRT-PCR

analysis to determine the changes in the transcript profiles of *PAL*, *C4H*, *4CL* and *STS* genes. As can be observed from Fig. 5, all the analyzed genes were induced by elicitation with CD and/or Cor. The expression of the *PAL* gene, which controls the first committed step of the *trans*-R biosynthetic pathway, was greatly enhanced by the presence of Cor at 8 h of treatment while the expression levels with CD alone or in combination with Cor were similar (Fig. 5). After 24 h of treatment, *PAL* transcript levels decreased, being the combined treatment which caused a greater increase. *PAL* and *STS* genes showed similar expression patterns (Fig. 5). The expression level of the *C4H* gene in *V. vinifera* SCC in response to CD separately or in combination with Cor was higher than that detected in the presence of Cor alone at 8 h of treatment while at 24 h, the elicitation with CD and Cor induced the highest levels. The highest expression levels of *4CL* gene, encoding the enzyme responsible for the formation of 4-coumaroyl-CoA, were found in the presence of CD and CD plus Cor at 8 and 24 h of treatments, respectively (Fig. 5). Therefore, although Cor induced the maximal levels of *PAL* and *STS* gene expression (Fig. 5), no significant amounts of *trans*-R were detected in the spent media. Such discrepancy could be due to either post-transcriptional and/or post-translational regulatory mechanisms. This fact was also observed by Lijavetzky et al. (2008) who showed that *STS* expression was highly induced by methyl jasmonate but no significant amounts of extracellular *trans*-R were detected. Similar studies were performed by Ramirez-Estrada et al. (2015) who analyzed the expression pattern of genes in *Taxus* SCC elicited with Cor and CD separately or in combination. In agreement with our results, these authors also observed that despite the fact that the expression of the two genes encoding enzymes directly responsible for the formation of taxol was higher in the cell cultures treated only with Cor, the highest production of this taxane was obtained under CD plus Cor elicitation. Consequently, they did not detect a clear relationship between the gene expression and the production of taxanes, and therefore, other factors may be involved.

In relation to expression of *C4H* and *4CL* genes, the results showed that the maximal levels were found with CD or CD plus Cor suggesting that the activity of these enzymes could be not only important for the formation of intermediates of *trans*-R biosynthesis but also for those intermediates involved in the biosynthesis of lignins and/or flavonoids. Lijavetzky et al. (2008) analyzed the effect of CD and methyl jasmonate on the relative expression of phenylpropanoid-related genes in *V. vinifera* SCC, when CD and methyl jasmonate were simultaneously added to the culture medium. They caused a significant increase in the gene expression of *PAL*, *C4H*, *4CL* and *STS* after 24 h of elicitation. Therefore, in this case the

effect of methyl jasmonate and Cor in the presence or absence of CD on gene expression seems to be similar.

4. Conclusion

The presence of Cor combined with CD in the culture medium dramatically increased *trans*-R production, being 942,8 and 3,7-fold greater than in the Cor and CD-treated SCC, respectively. In fact, the levels of *trans*-resveratrol produced in the combined treatment were higher than the sum of the individual treatments, and therefore we observed a synergistic effect using CD and Cor. In addition, our experiments provide new information of the effect of Cor on grapevine cell cultures since it is the first time that this elicitor is used in combination with CD which potentially are inducers of *trans*-R biosynthesis. Moreover, both elicitors induced a high reprogramming of gene expression of *V. vinifera* SCC, which likely accounts for enhancing the production of *trans*-R. In addition, all the analyzed genes were induced by CD and/or Cor being the expression maximal of the *PAL* and *STS* in the presence of Cor, and the expression levels of *C4H* and *4CL* in the presence of CD. Our study showed the possibility of increasing *trans*-R production and secretion from the producer cells to culture medium by the addition of CD with this new elicitor, Cor.

Contribution

LA, RB and MAP conceived and designed research. LA, SBB and AMM conducted experiments. LA, AMM and MAP analyzed data. LA and MAP wrote the manuscript. All authors read and approved the manuscript.

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ACCEPTED MANUSCRIPT

Figure Captions

Fig. 1 Effect of 50 mM CD separately or in combination with 0,5, 1 or 2 μ M Cor on extracellular production of *trans*-R in grapevine SCC after 96 h of treatments. Experiments were repeated three times. Data are the mean \pm SD of the replicates.

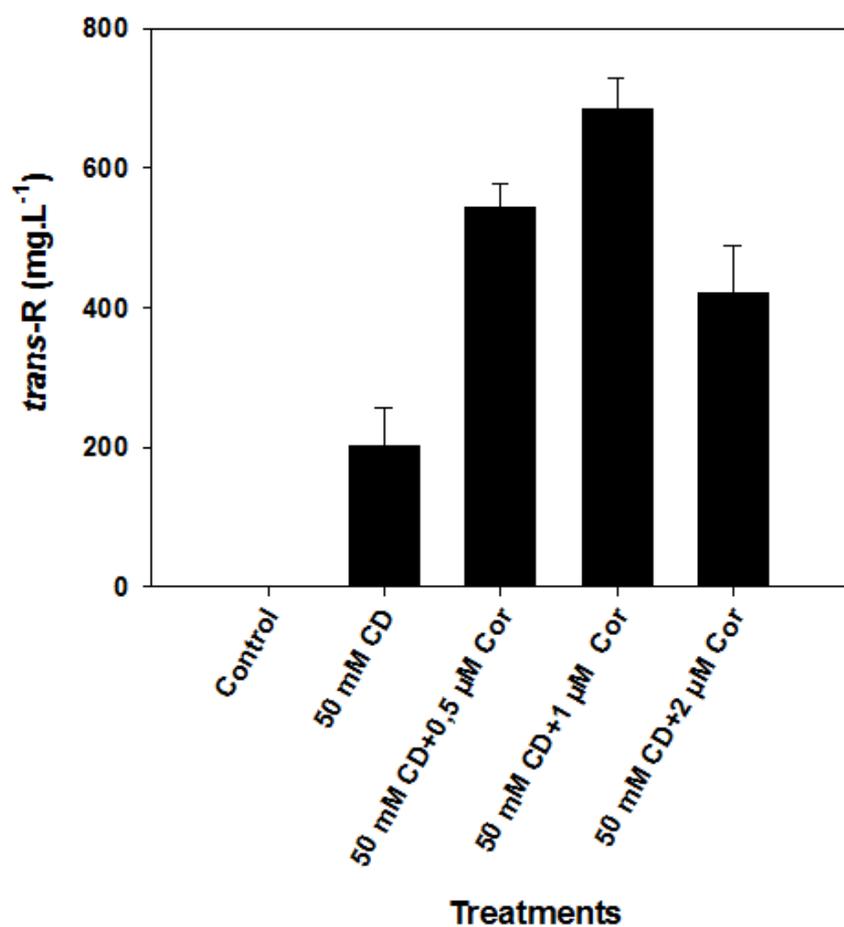


Fig. 2 Effect of elicitation time course on extracellular production of *trans*-R in grapevine SCC treated with 50 mM CD and/or 1 μ M Cor after 168 h of treatments. Experiments were repeated three times. Data are the mean \pm SD of the replicates.

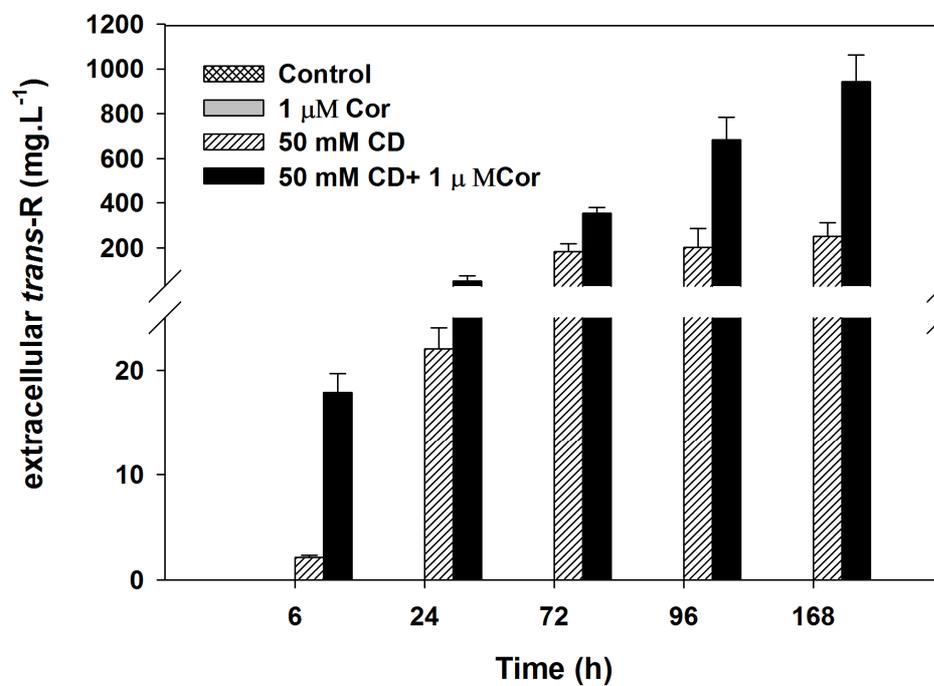


Fig. 3 Effect of elicitation time course on intracellular production of *trans*-R in grapevine SCC treated with 50 mM CD and/or 1 μ M Cor after 168 h of treatments. Experiments were repeated three times. Data are the mean \pm SD of the replicates.

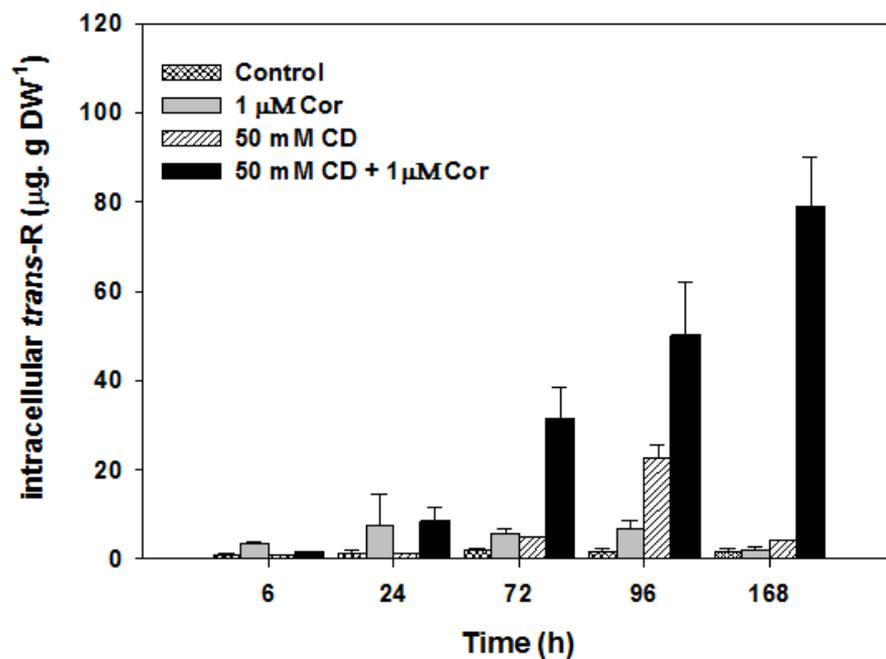


Fig. 4 Effect of different elicitors on cell growth measured as g DW. L⁻¹ in *V. vinifera* SCC after 168 h of elicitation. Experiments were repeated three times. Data are the mean \pm SD of the replicates.

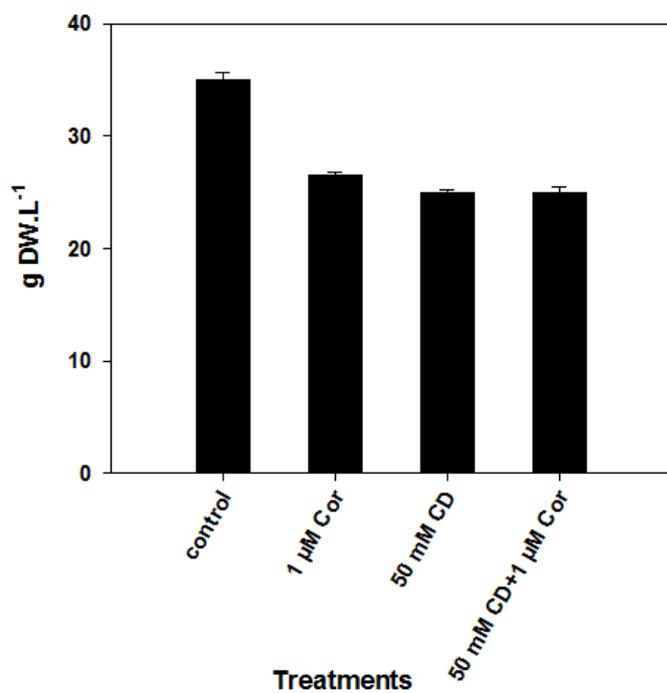
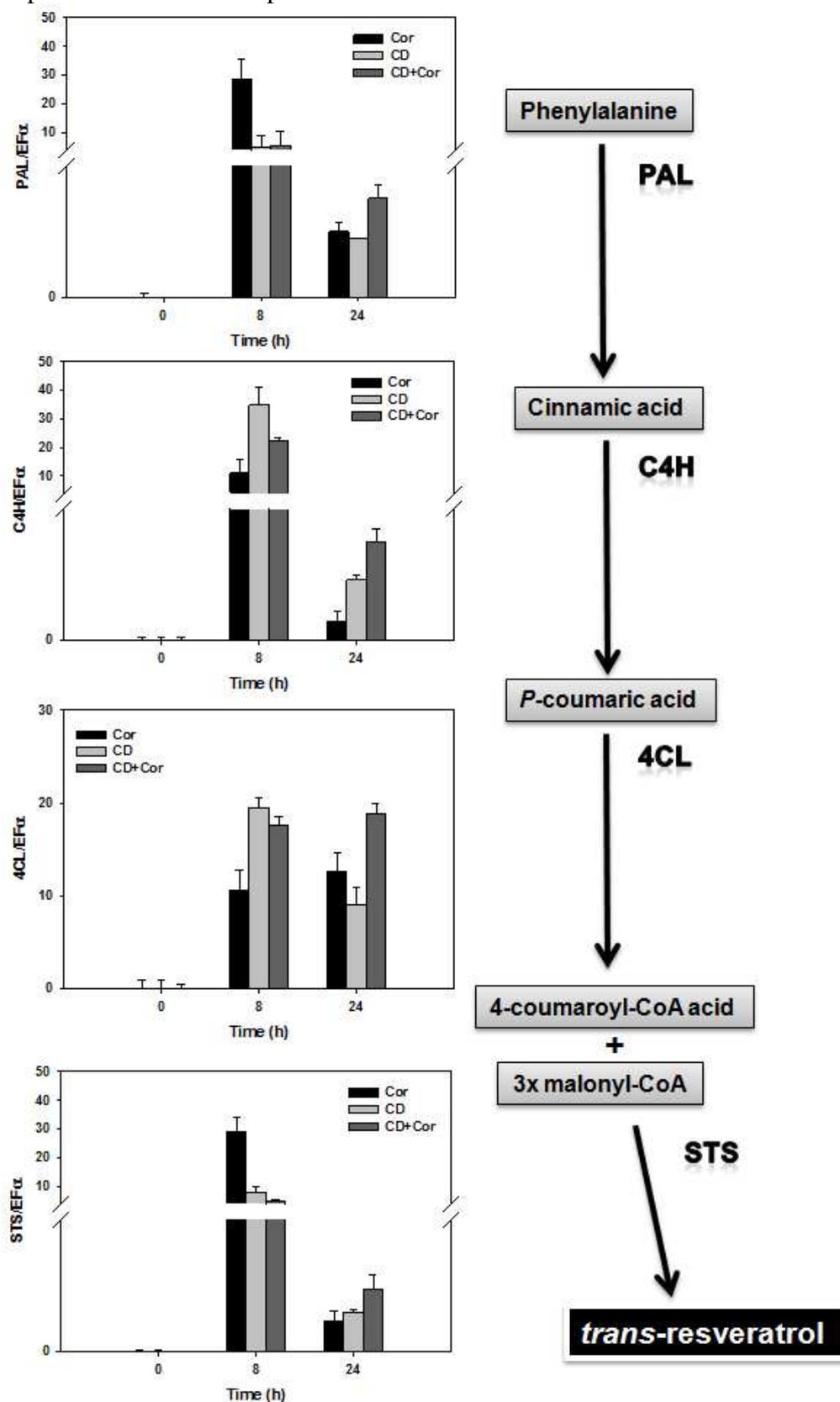


Fig. 5 Relative expression of the *PAL*, *C4H*, *4CL* and *STS* genes in *V. vinifera* SCC elicited with 50 mM CD and/or 1 μ M Cor at 0, 8 and 24 h of treatment. Levels of transcripts were calculated using the EF- α gene as internal control. Values are given as the mean \pm SD of one experiment with three replicates.



Highlights

The maximum level of resveratrol was reached with cyclodextrins and 1 μ M coronatine.

The combined treatment induces a synergistic effect of resveratrol production.

Coronatine increases the levels of *phenylalanine ammonia lyase* and *stilbene synthase*.

Cyclodextrins increases the levels of *cinnamate hydroxylase* and *coumarate-CoA ligase*.