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DOI: 10.2225/vol15-issue5-fulltext-4 *RESEARCH ARTICLE*

Stimulation of *trans*-resveratrol biosynthesis in *Vitis vinifera* cv. Kyoho cell suspension cultures by 2, 3-dihydroxypropyl jasmonate elicitation

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Abstract

Background: Plant cell suspension culture of *Vitis vinifera* is a promising technology for investigating different factors that are able to induce and/or modify stilbenes biosynthesis. Jasmonates have been reported to play an important role in a signal transduction pathway that regulates defence responses as well as the production of secondary metabolites. In this study, 2, 3-dihydroxypropyl jasmonate (DHPJA) was used to investigate its effect on stimulating *trans*-resveratrol (*t*-R) accumulation and the plant defence responses in *Vitis vinifera* cv. Kyoho cell suspension cultures for the first time. **Results:** It demonstrated that DHPJA had superior effects on stilbenoids accumulation over methyl jasmonate (MeJA). The optimal condition was 150 μM DHPJA added on day 15 of cultivation period, with the highest level of *t*-R accumulation which was increased 1.8-fold and 1.3-fold compared with the control and 150 μM MeJA respectively. DHPJA induced stronger plant defence responses, including oxidative burst and activation of L-phenylalanine ammonia lyase (PAL) than MeJA. H_2O_2 generation induced by DHPJA played a significant role in enhancing *t*-R accumulation. Adding a specific inhibitor of H_2O_2 signalling pathway inhibited DHPJA-induced *t*-R accumulation, but had no effects on DHPJA-induced other metabolites accumulation, which resulted in regulations of product diversity. **Conclusions:** This study demonstrated that DHPJA was an efficient elicitor to enhance *t*-R accumulation by activating stronger oxidative burst, and H_2O_2 signalling pathway could regulate product diversity in DHPJA-induced *V. vinifera* cv. Kyoho cell suspension cultures.

Keywords: 2, 3-dihydroxypropyl jasmonate, H_2O_2, *trans*-resveratrol, *Vitis vinifera* cell suspension cultures

INTRODUCTION

Trans-resveratrol (*t*-R, 3, 5, 4'-trihydroxy-*trans*-stilbene), belonging to the stilbenoids family, possesses numerous important bioactivities, including anti-inflammatory, antioxidant, anti-aggregatory functions, and modulation of lipoprotein metabolism (Shankar et al. 2007). And it has been shown to possess chemo preventive properties against certain cancers and cardiovascular diseases and to have positive effects on age longevity (Aggarwal et al. 2004; Shankar et al. 2007). Now the demand for *t*-R is growing every day. However, supplying from extracts of intact plant is insufficient. Extensive effort has been prompted to develop alternate sources and means for *t*-R production.

Plant cell culture is a useful tool for producing active secondary metabolites under controlled conditions. Due to the high level of stilbenes accumulation in grape, some authors have undertaken studies on grapecell cultures to investigate different factors that are able to induce and/or modify

stilbenes biosynthesis, regulation and metabolism (Krisa et al. 1999; Commun et al. 2003). New strategies based on the use of *Vitis vinifera* cell cultures have been used to increase *t*-R production (Donnez et al. 2009; Kiselev, 2011). Different elicitors have been used, such as fungal cell wall fragments (Liswidowati et al. 1991), cyclodextrins (CDs) (Bru and Pedreño, 2003; Bru et al. 2006), Na-orthovanadate, laminarin (Aziz et al. 2003), chitin (Yang et al. 2010), jasmonic acid (JA) and its more active derivative, methyl jasmonate (MeJA) (Tassoni et al. 2005; Vezzulli et al. 2007; Belhadj et al. 2008; Lijavetzky et al. 2008; Donnez et al. 2011).

Jasmonates have been reported to play an important role in a signal transduction pathway that regulates expression of defence genes in plants. In these cases, the production of secondary metabolites changed dramatically in response to elicitation (Qian et al. 2004; Hu et al. 2006). JA and MeJA have been proved to be effective in stimulating stilbenoids biosynthesis in different cell lines of *Vitis* spp. (Tassoni et al. 2005; Belhadj et al. 2008; Donnez et al. 2011, Kiselev, 2011). MeJA up-regulated the gene expression of defence-related proteins (PR protein) as well as key enzymes in the phenylpropanoid pathway via which stilbenoids are synthesized. However, it was unclear whether jasmonates elicited *t*-R biosynthesis via a specific signal transduction pathway.

Compared to MeJA, chemically synthesized 2, 3-dihydroxypropyl jasmonate (DHPJA) was demonstrated to have superior effects on taxane, hypericins and hyperforin production (Qian et al. 2004; Liu et al. 2007). It acted as a powerful signal molecular to activate stronger plant defence responses, including oxidative burst and activation of PAL in *Taxus chinensis* suspension cultures (Qian et al. 2004). But in *Panax notoginseng* cell cultures, oxidative burst might not be involved in the jasmonates-elicited signal transduction pathway. MeJA and 2-hydroxyethyl jasmonate (HeJA) induced the ginsenoside biosynthesis via induction of endogenous JA biosynthesis and key enzymes, such as UDPG-ginsenoside Rd glu-cosyltransferase (UGRdGT), in the ginsenoside biosynthetic pathway (Wang et al. 2006). However, the application of DHPJA in other plant cell cultures is rare. Its effect and mechanism of enhancing stilbenoids synthesis has not been studied. And it's uncertain whether oxidative burst is involved in the DHPJA-elicited signal transduction pathway in *V. vinifera* cell cultures.

This study compared the effects of MeJA and DHPJA on inducing *t*-R accumulation in *V. vinifera* cell suspension cultures, and investigated the role of H_2O_2 in enhancing stilbenoids synthesis and activating of PAL. Meanwhile, using a specific inhibitor of H_2O_2 signalling pathway to regulate product diversity was firstly investigated in DHPJA-induced *V. vinifera* cv. Kyoho cell cultures.

MATERIALS AND METHODS

Grape callus induction and cell cultures

The callus, induced from the peel of mature berries of healthy *Vitis vinifera* cv. Kyoho in later ripening stage, were cultivated on Murashige and Skoog (MS) medium containing 6 mg/L 1-naphthlcetic acid (NAA), 0.6 mg/L 6-benzyladenine (BA), 30 g/L sucrose and 0.9% (w/v) agar at 25 ± 2ºC under dark. Cell suspension cultures were maintained in liquid MS medium containing 30 g/L sucrose and the same growth regulator combination in 50 mL Erlenmeyer flasks on an orbital shaker at 110 rpm at 25 ± 2ºC under dark. Each of the cultures flasks was filled with 10 mL medium and inoculated with 1 g fresh cells from the solid cultures.

Elicitation experiments

MeJA and DHPJA were supplied by Institute of Pesticides and Pharmaceuticals, East China University of Science and Technology. They were dissolved in ethanol at the concentration of 0.04 mM for stock.

To determine the optimal eliciting time, 150 μM of DHPJA (final concentration) was added on different days (Figure 1). To determine the optimal concentrations of elicitors, different concentrations of MeJA and DHPJA (Figure 2) were added on day 15, and samples were harvested on day 20.

To inhibit the induction of H_2O_2 generation, 10 μM NADPH oxidase inhibitor (DPI, diphenyleneiodonium chloride, Sigma) was added 30 min before elicitation.

Control received no treatment. It was confirmed that 1‰ (v/v) ethanol had no side effects on cell growth, *t*-R accumulation, H_2O_2 production, or PAL activity (data not shown).

Determination of cell mass and *trans*-resveratrol accumulation

The cell suspension was filtered under vacuum. The biomass was dried at 45ºC to constant weight. And the dry weight (DW) was determined.

For the extraction of intracellular *t*-R, 100 mg dried cells were powdered and dissolved in 1 mL methanol, and the sample was extracted by ultrasonic for 2 times, 30 min per time. Subsequently, the extract was filtered through a 0.22 μm nylon filter and analyzed in a HPLC-DAD system using a reversed phase C18 column (4.6 mm x 250 mm, 5 μm, Agilent ZORBAX Eclipse XDB-C18) at 25ºC. The mobile phase was acetronitrile: water (30:70, v/v) with flow rate of 1 mL/min and the injection volume was 20 μL. The content of *t*-R was given on a standard curve made with *trans*-resveratrol standard (Sigma). The samples were also identified by electrospray ionisation mass spectra (ESI/MS) at the Center of Analysis, Shanghai Jiao Tong University. A mass spectrometer (Waters, USA) was used with sheath gas flow rate of 50 arb, sweep gas flow rate of 20 arb, spray voltage of 3.0 kV and capillary temperature of 300ºC. The spectrum was scanned at 30 eV from m/z 50 to 600.

Assay of oxidative burst and PAL activity

H_2O_2 generated in the cells and released into medium was determined according to Sergiev et al. (1997). Briefly, 0.5 mL sample medium was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7) and 1 mL 1 M KI directly. The reaction mixture was incubated for 1 hr at 25ºC under dark. The absorbance of the mixture was read at 390 nm before and after incubation. The content of H_2O_2 was given on a standard curve.

To determine the activity of PAL, 1 g fresh cells were extracted with 2 mL 50 mM pH 8.8 Tris-HCl buffer containing 4 mM of β-mercaptoethanol and 10 mM EDTA in an ice-cooled mortar. The homogenate was centrifuged at 10000 rpm for 15 min at 4ºC, and the supernatant was collected for enzyme assay. PAL activity was determined based on the rate of cinnamic acid production as described by Ochoa-Alejo and Gómez-Peralta (1993). One unit of PAL activity is equal to 1 μmol of cinnamic acid produced per minute.

Statistical analyses

Each experiment was carried out in triplicate, and three different sets of experiments reproduced the same result. Data are presented as mean ± standard deviations (S.D.) Statistical significance was determined using the paired Student's *t* test.

RESULTS

Effects of elicitation timing and dosage on cell growth and *t*-R production

Figure 1 showed that day 15 after inoculation was the optimal eliciting time. When the treatment was done on that day, the highest production of *t*-R was observed on day 20 (5 days after the treatment), which was increased 1.96-fold compared with the control. Cell growth was strongly suppressed by 65-84%, and the *t*-R production couldn't be stimulated when elicited in earlier phases. Eliciting in a later phase was also less efficient, although the *t*-R production was increased 1.54-fold compared with the control. Figure 2 showed that 150 μM was the optimal eliciting concentration for both DHPJA and MeJA. Adding an excessive dose depressed cell growth by 47% (Figure 2a), while adding lower doses lowered the elicitation effect (Figure 2b and 2c). It also showed that DHPJA was more efficient in inducing *t*-R accumulation than MeJA. When the adding concentration was 150 μM, the *t*-R production induced by DHPJA increased 1.3-fold compared with MeJA.

Effects of DHPJA on plant cell defence responses

Figure 3 showed that the H_2O_2 level changed markedly when treated with DHPJA or MeJA. The H_2O_2 level reached its maximum at 45 min after exposure to DHPJA (5.04 ± 0.36 μM), which was higher than exposure to MeJA (3.94 ± 0.49 μM). Figure 4 showed that PAL activity in elicited cultures increased significantly at 4 hrs after elicitation, and increased to approximately 4.6-fold at 6 hrs after elicitation, which was also higher than MeJA-elicited cultures.

Effects of H_2O_2 on DHPJA-induced stilbenoids accumulation

Figure 3 showed that diphenyleneiodonium chloride (DPI) successfully inhibited the DHPJA-induced generation of H_2O_2 by 90.31%. Correspondingly, DPI inhibited DHPJA-induced *t*-R content by 89.18% (Figure 5 and Figure 6a). Meanwhile, DPI and H_2O_2 also had impact on the activity of PAL. Data was shown in Figure 4. DPI inhibited the DHPJA-induced activity of PAL by 43.37%. And adding H_2O_2 significantly stimulated the PAL activity by itself and enhanced the DHPJA elicitation effect. Correspondingly, Figure 5 and Figure 6a showed that adding H_2O_2 stimulated *t*-R production by itself and also enhanced the DHPJA elicitation effect.

In Figure 6b, peak 1 was demonstrated to be *t*-R. Peak 2 was another compound which was conjectured to be a stilbenoid analogue by profiles of full wavelength scan and mass spectrometer (MS) (Figure 6b and Figure 7). It also could be induced by DHPJA, but the induction could not be inhibited by DPI (Figure 6a).

DISCUSSION

Effects of elicitation timing and dosage on cell growth and *t*-R production

Elicitation timing and dosage are the two main variables that affect cell growth and yield of secondary metabolites (Wang et al. 2006). It appears that plant cells responded differently to elicitation timing and dosage, which may be attributed to the differences not only among plant cell species but also among cell lines within species. In micro shoots of *Hypericum perforatum* 100 μM DHPJA enhanced hyperforin production stronger than 50 μM DHPJA, while it was the opposite in micro shoots of *Hypericum sampsonii* (Liu et al. 2007). In our culture system, addition of 150 μM DHPJA on day 15 of cultivation period was found to be optimal. The *t*-R production reached the highest value which was increased 1.8-fold and 1.3-fold compared with the control and 150 μM MeJA respectively.

It is speculated that when a dose lower than optimal is used, the elicitor-interaction sites in cells are not sufficiently utilized for activating the secondary metabolite synthesis, whereas an excessive dose causes cytotoxic effects (Lambert et al. 2009). It also indicated that the response of *t*-R biosynthesis to elicitation timing was closely related to the cellular physiological state. In conclusion, adding excessive doses and/or eliciting in earlier physiological states depressed cell growth, while adding lower doses and/or eliciting in later physiological states lowered the elicitation effect.

DHPJA-induced defence responses and their effects on *t*-R production

DHPJA activated stronger defence responses including oxidative burst and activity of PAL in *V. vinifera* cell suspension cultures, corresponded well with its superior stimulating activity for *t*-R biosynthesis, compared with MeJA. The results suggested that an oxidative burst occurred in elicited *Vitis vinifera* cell cultures in a manner similar to that seen in other elicited plant cell cultures (Qian et al. 2004). There is an increasing body of evidence that H_2O_2 can act as a diffusible signal to activate defence genes and the biosynthesis of plant secondary metabolites (Jabs et al. 1997).

Our work indicated that H_2O_2 played a significant role in *t*-R biosynthesis. However, while the DHPJA-induced *t*-R accumulation was totally inhibited by DPI, the activity of PAL remained higher than control. It suggested that DHPJA also induced the activation of PAL through other molecular pathways to enhance other metabolites synthesis, which might correspond with the accumulation of peak 2. In *Taxus yunnanensis* suspension cells, NO enhanced cerebroside-induced activation of PAL and

accumulation of taxol (Wang et al. 2007). Although PAL is a key enzyme in the phenylpropanoid pathway, the activation induced by DHPJA could not result in *t*-R accumulation without H_2O_2. So inducing higher level of H_2O_2 generation was the basic reason why DHPJA could enhance *t*-R accumulation stronger than MeJA. To our best knowledge, this was the first report to investigate the relationship between H_2O_2 and stilbenoids synthesis.

In conclusion, our work demonstrated that DHPJA was an efficient elicitor for *t*-R accumulation by activating stronger oxidative burst. It is believed that by taking the place of MeJA, DHPJA might optimize other strategy for *t*-R production. It also indicated that H_2O_2 signalling pathway could regulate product diversity in DHPJA-induced *V. vinifera* cell suspension cultures.

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Figures

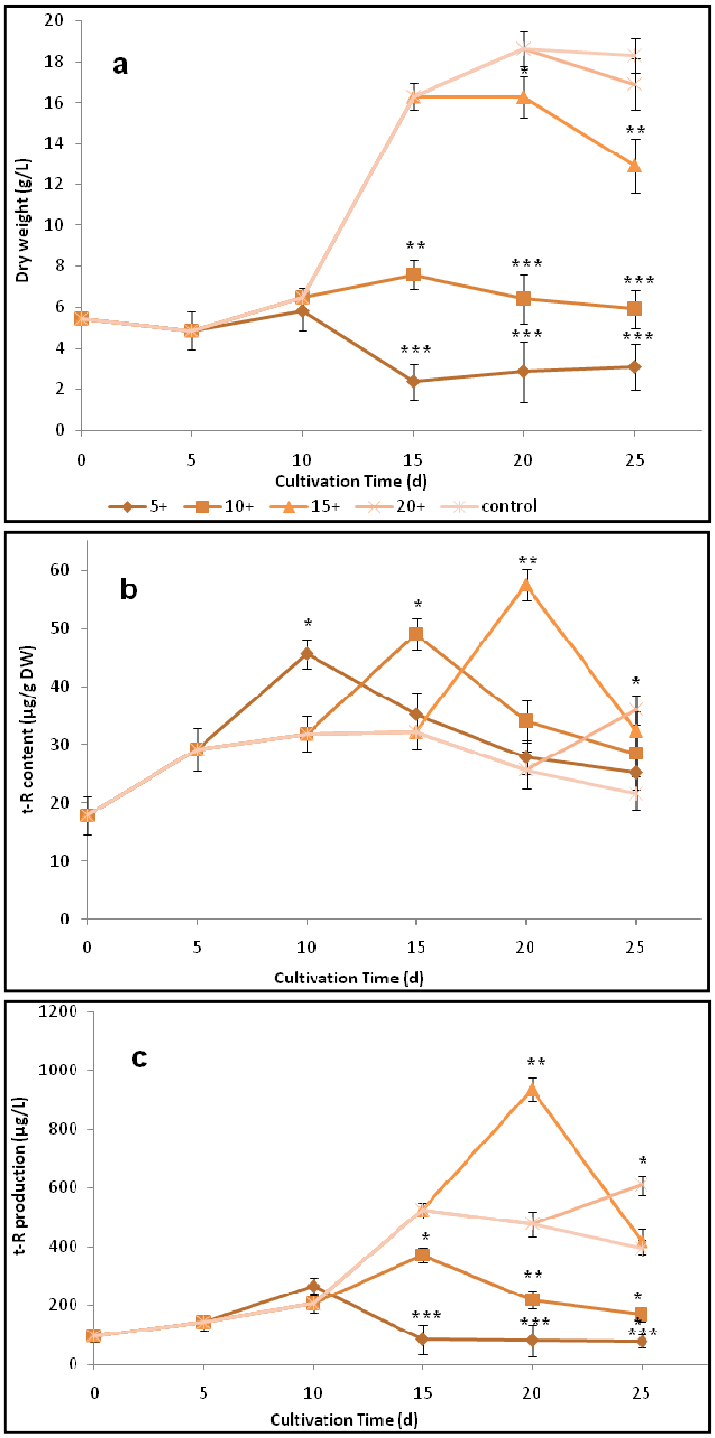

Fig. 1 Time courses of cell growth and *t*-R accumulation in *V. vinifera* cell suspension cultures elicited with DHPJA on different days: (a) Dry cell weight; (b) *t*-R content; and (c) *t*-R production. In the figure, 5+, 10+, 15+ and 20+ mean that DHPJA (150 µM) was added on day 5, 10, 15 and 20. Data are the means of three flasks and vertical bars show standard deviations. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, significantly different from Control.

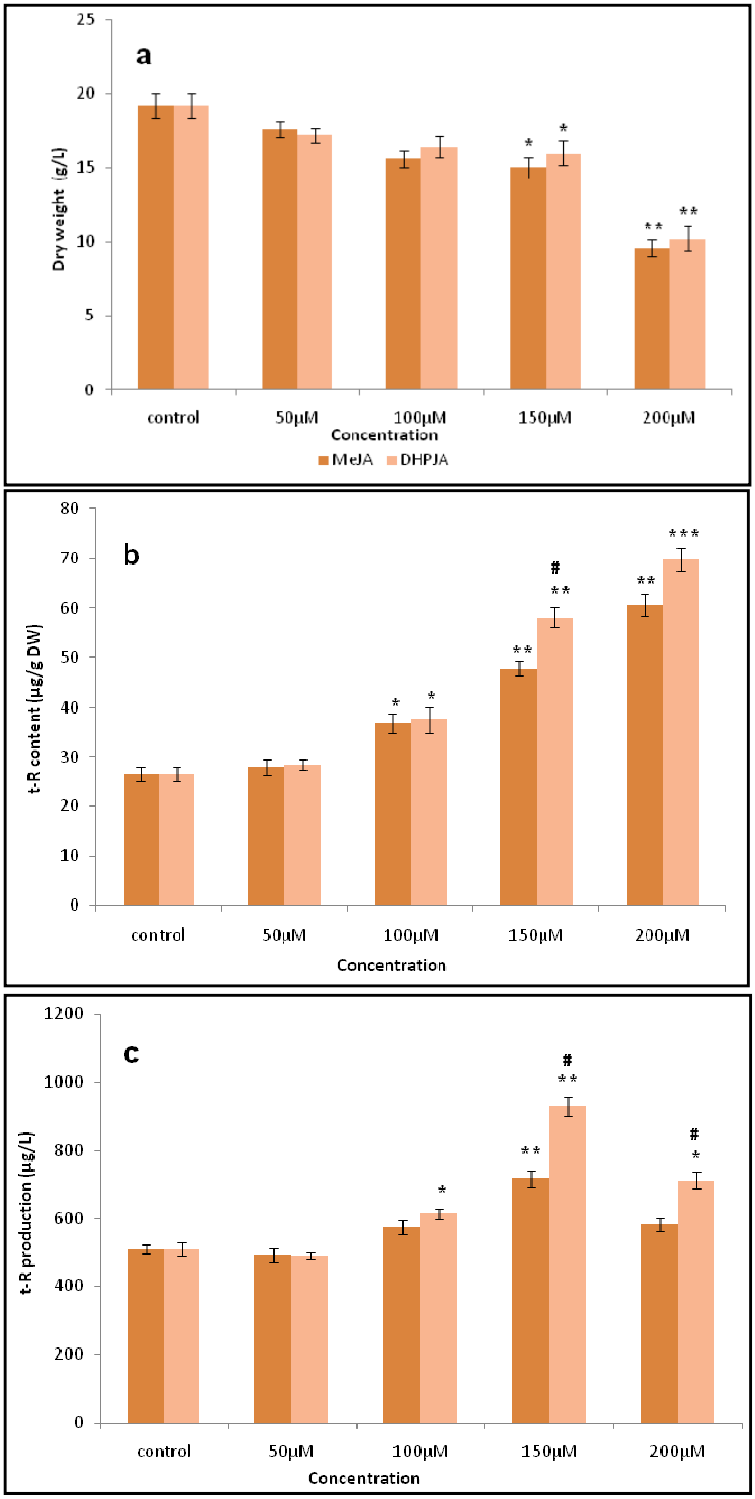

Fig. 2 Effect of DHPJA and MeJA with various concentrations on cell growth and *t*-R accumulation in *V. vinifera* cell suspension cultures: (a) Dry cell weight; (b) *t*-R content; (c) *t*-R production. Data are the means of three flasks and vertical bars show standard deviations. *, P < 0.05, **, P < 0.01, ***, P < 0.001, significantly different from Control. #, P < 0.05, significantly different from MeJA.

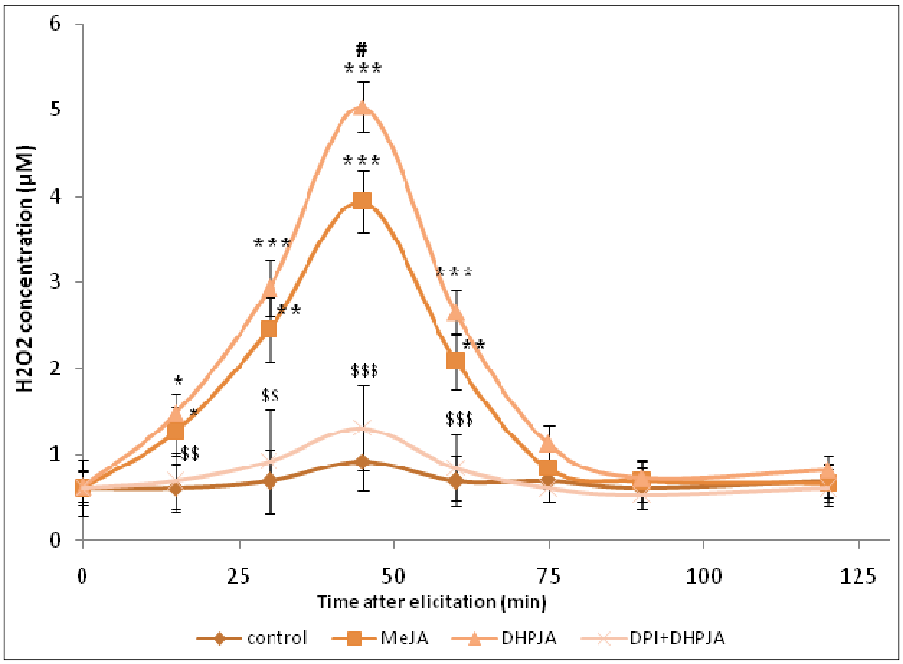

Fig. 3 The oxidative burst induced by DHPJA and MeJA DHPJA (150 µM) and MeJA (150 µM) were added on day 15. DPI+DHPJA means DPI (10 µM) was added 30 min before DHPJA. Data are the means of three flasks and vertical bars show standard deviations. *, P < 0.05, **, P < 0.01, ***, P < 0.001, significantly different from Control. #, P < 0.05, significantly different from MeJA. $, P < 0.05, $$, P < 0.01, $$$, P < 0.001, significantly different from DHPJA.

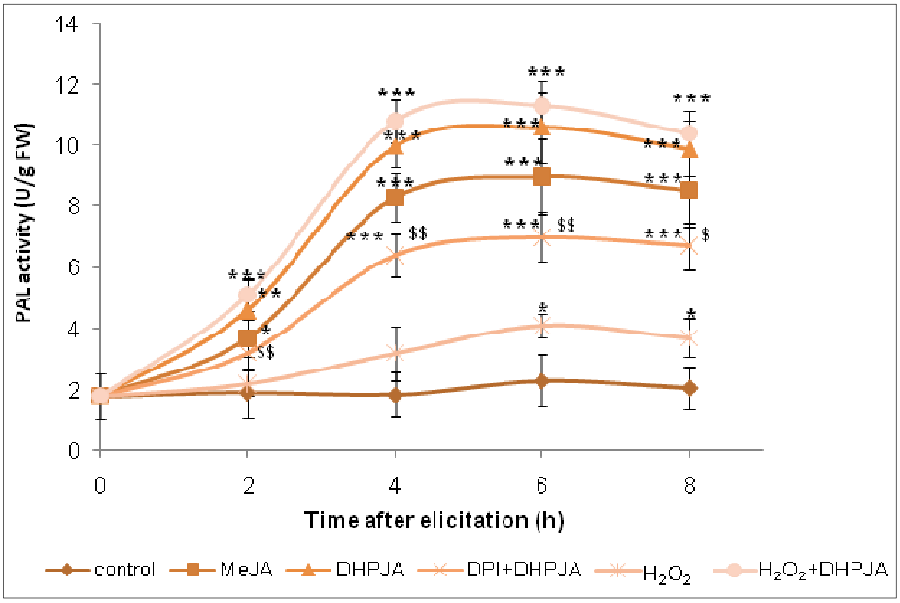

Fig. 4 PAL activity in response to different treatments DHPJA (150 μM) and MeJA (150 μM) were added on day 15. DPI+DHPJA means DPI (10 μM) was added 30 min before DHPJA. H_2O_2+DHPJA means 5 μM H_2O_2 was added with DHPJA. Data are the means of three flasks and vertical bars show standard deviations. *, P < 0.05, **, P < 0.01, ***, P < 0.001, significantly different from control. #, P < 0.05, significantly different from MeJA. $, P < 0.05, $$, P < 0.01, $$$, P < 0.001, significantly different from DHPJA.

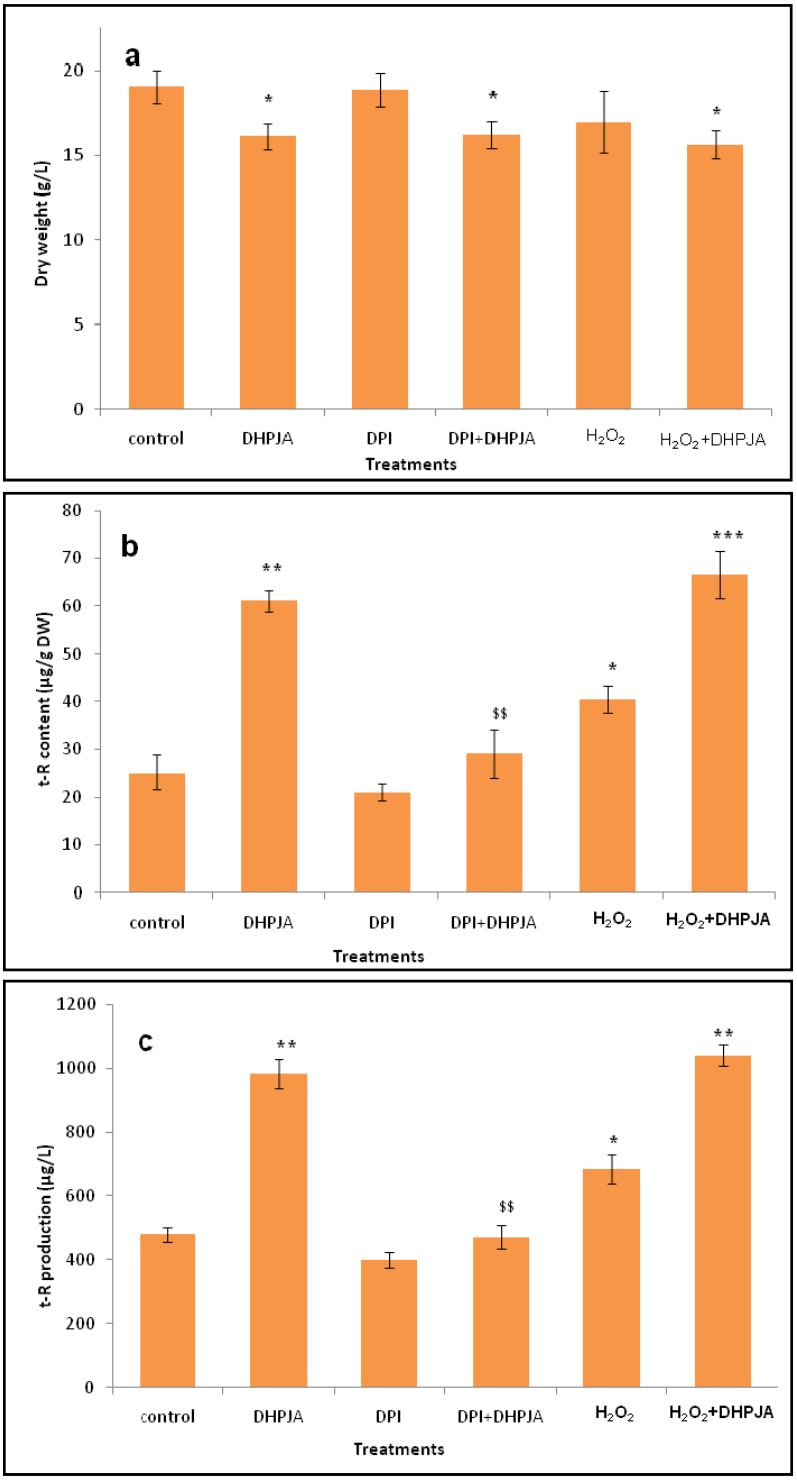

Fig. 5 Effect of H_2O_2 on cell growth and *t*-R accumulation in *V. vinifera* cell suspension cultures: (a) Dry cell weight; (b) *t*-R content; and (c) *t*-R production. Cell suspension cultures was elicited with different treatments on day 15. And samples were harvested on day 20. Data are the means of three flasks and vertical bars show standard deviations. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, significantly different from Control. $$, $P < 0.01$, significantly different from DHPJA.

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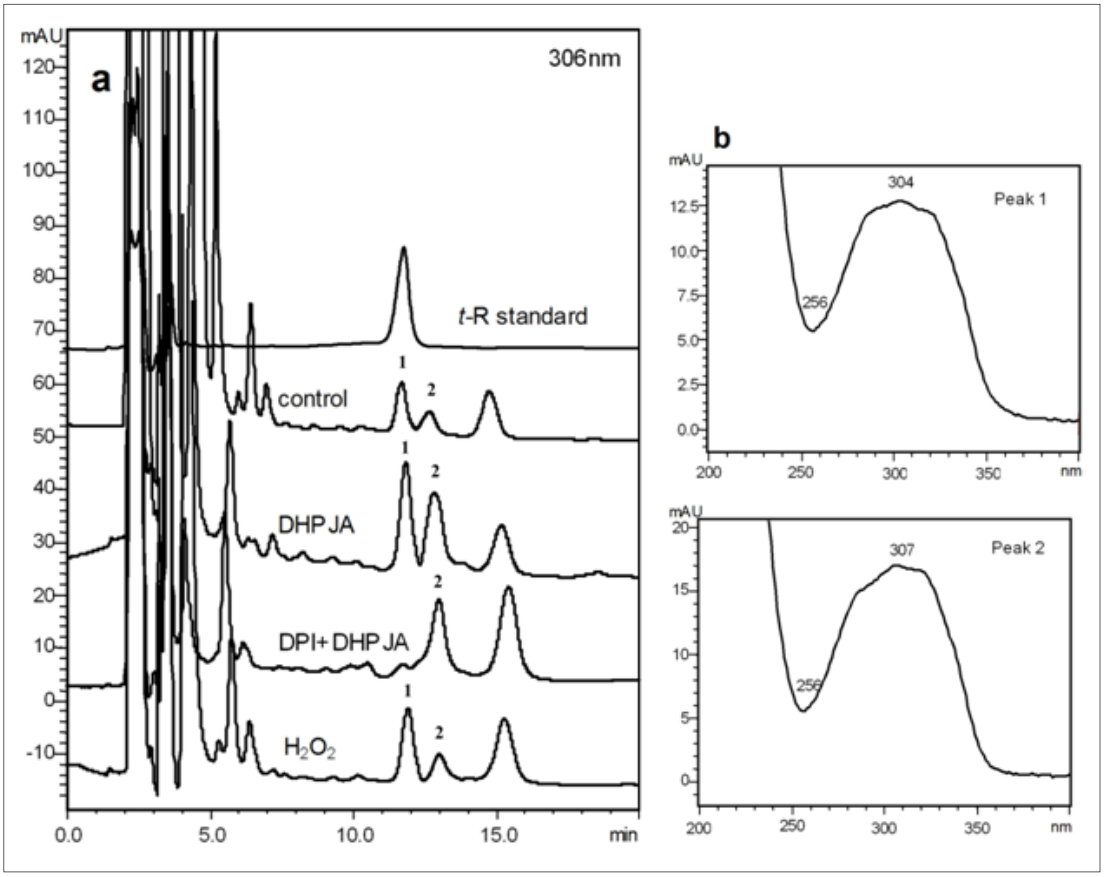

Fig. 6 HPLC profiles (a) of cultures with different treatments, and full wavelength scan profiles (b) of peak 1 and peak 2.

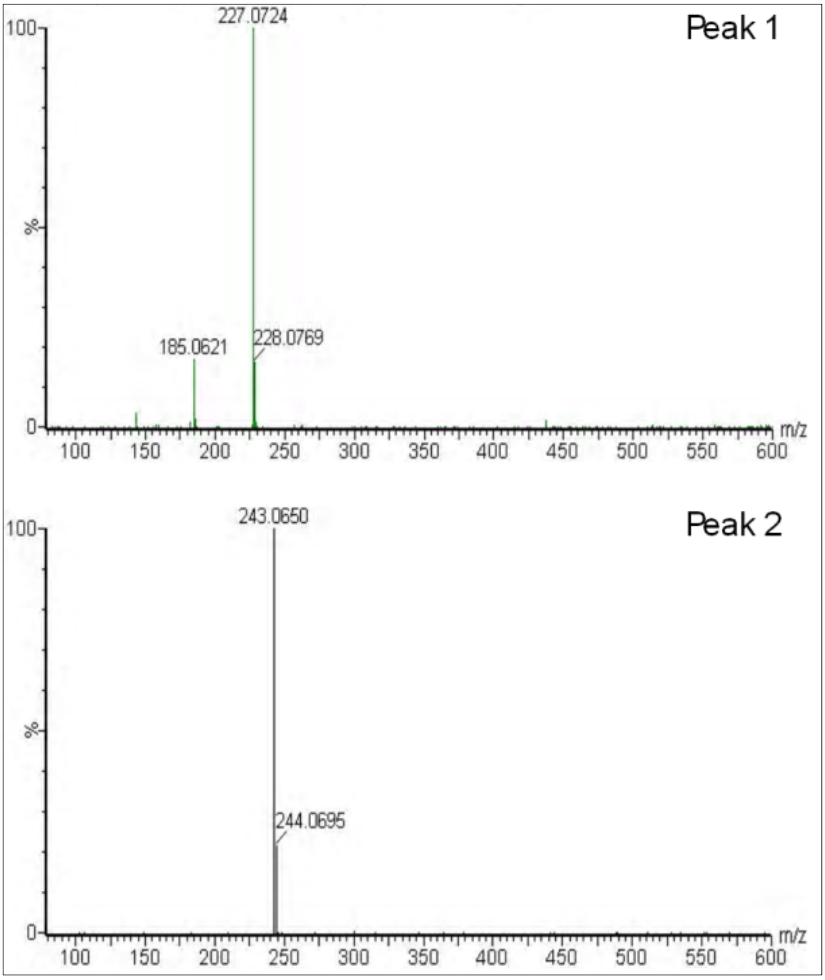

Fig. 7 MS profiles of peak 1 and peak 2.