

Ultra Violet Irradiation Enhances Resveratrol Production in Organs and Cell Suspension Cultures of Two Iranian Grape (*Vitis vinifera* L.) Cultivars

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ABSTRACT

Resveratrol (3,5,4'-trihydroxystilbene) is a valuable aromatic compound that prevents cancer and coronary heart diseases in human. In our previous study, this compound was primarily extracted from fruits of 147 Iranian grape cultivars and determined by high performance liquid chromatography (HPLC). Of these cultivars, two of the most desirable ones ('Rajabi Sefid Shiraz' and 'Keshmeshi Ghermez') were selected for the present study and the amount of resveratrol in their leaves and fruits was measured. The amount of this compound in fruits of both cultivars was more than their leaves and those treated with UV ray ($\lambda = 254$ nm) also produced more resveratrol than the controls. Callus and cell suspension cultures of leaves and fruits of these cultivars were established and the production of resveratrol in their cells was determined. Callus was produced from leaf and fruit explants of the two cultivars 4 and 6 weeks after culture, respectively. Cells derived from the callus of both explants established in suspension cultures after three subcultures and produced resveratrol. Cells were exposed to UV 6 days after the third subculture when they entered into an exponential growth stage. The amount of resveratrol produced by UV-treated cells was significantly higher than the organs as well as the controls.

Keywords: aromatic compound, callus, Keshmeshi Ghermez, Rajabi Sefid Shiraz

INTRODUCTION

Resveratrol (3,5,4'-trihydroxystilbene) is a low molecular weight polyphenolic compound belonging to a group of phytoalexins, the stilbenes (Langcake and Pryce 1977). Stilbenes play an important role in plants to defend against pathogens, especially fungi (Dixon and Harrison 1990; Hain *et al.* 1990; Jeandet *et al.* 2002). Resveratrol and picied are two of the most important stilbenes forming the chemical structure of aromatic compounds with both *cis* and *trans* isomers. Plants normally produce more *trans*-resveratrol than the *cis* isomer. Picied is also a glucosylated isomer of resveratrol that is highly accumulated in some plants such as grapes, producing stilbenes (Krasnow and Murphy 2004).

Among the plant families, Vitaceae, Pinaceae and Arachnaceae produce resveratrol when exposed to biotic and physiochemical stresses. Grapevine (*Vitis vinifera*) and peanut (*Arachis hypogaea*) are the most important plants producing resveratrol and grapes are the main source of this compound in the human diet (Langcake and Pryce 1976; Jeandet *et al.* 1995; Kodan *et al.* 2001; Krasnow and Murphy 2004; Tassoni *et al.* 2005). Resveratrol has substantial biological benefits for humans; it prevents the growth of cancerous cells and reduces the risk of coronary heart and vascular diseases (Frankel *et al.* 1995; Folts 2002; Delmas *et al.* 2006). This compound is highly accumulated in fruits, leaves and stems of grape plants in response to biotic and non-biotic stresses (Jeandet *et al.* 2002; LeBlanc 2006).

Many studies have so far been conducted regarding the effect of ultra violet (UV) irradiation ($\lambda = 254$ nm in all reports) on the production and accumulation of resveratrol in grape cells. Langcake *et al.* (1977) and Roggero and Garcia-Parrilla (1995) reported that UV rays stimulated both the production and accumulation of this compound in grapes. UV irradiation, visible light and methyl jasmonate have

been the most important physiochemical treatments used to produce resveratrol in grape cell suspension culture systems. Synthesis of stilbenes in grapes and peanuts involving stilbene synthase, a key enzyme of resveratrol biosynthesis, is stimulated by treatment with UV and methyl jasmonate (Fritzemeier and Kindl 1981; Douillet-Breuil *et al.* 1999; Tassoni *et al.* 2005; Medina-Bolivar *et al.* 2007; Tang *et al.* 2010) and causes stilben synthase gene(s) to be expressed (Versari *et al.* 2001; Grimming *et al.* 2002; Chung *et al.* 2003; Chong *et al.* 2009; Condori *et al.* 2009). A significant increase in resveratrol content of grape cells has been reported by Cantos *et al.* (2000, 2001) and Jeandet *et al.* (2002) when they exposed cells to UV. Krasnow and Murphy (2004) investigated the effects of UV irradiation together with visible light, only visible light and dark on resveratrol accumulation in grape cells and observed that glucosyl-transferase enzyme activity, resveratrol and the resveratrol glucosylated isomer (Picied) were produced more in the dark than in other treatments, and that the activity of this enzyme decreased significantly in visible light. They have also found that picied accumulation was greatly reduced in cells treated with UV plus visible light compared with those exposed to only visible light, and finally, the UV-treated cells produced a considerable amount of resveratrol. The effects of UV irradiation period, incubation period and callus age have also been investigated for *trans*-resveratrol induction in callus cultures of grapes by Keskin and Kunter (2008, 2009, 2010). Their results showed that calli treated with UV irradiation could be induced, being a convenient method for resveratrol production. Distribution of resveratrol and stilbene synthase in young grape plants have been studied by Wang *et al.* (2010) when they subjected grape leaves to UV irradiation and found that resveratrol and stilbene synthase were both intensely stimulated in grape leaves, with a similar response pattern.

In this study, the amount of *trans*-resveratrol in leaves,

fruits and cells of two Iranian grape cultivars was determined. UV ray-treated organs and suspension cultures were also evaluated for the production of this compound.

MATERIALS AND METHODS

In our previous study, resveratrol was primarily extracted from fruits of 147 Iranian grape cultivars and determined by High Performance Liquid Chromatography (HPLC). Of these cultivars, two of the most desirable ones ('Rajabi Sefid Shiraz' and 'Keshmeshi Ghermez') were selected for the present study and the amount of resveratrol in their leaves and fruits was measured. Adult leaves and fruits of both cultivars were also exposed to UV light ($\lambda = 254$ nm) for 12 h. Extraction of phenolic compounds from the samples and determination of resveratrol were carried out as described in Esna-Ashari *et al.* (2008).

In order to prepare explants for callus production, leaves and fruits of both cultivars were washed under tap water to remove dust and sterilised by emerging in 5% sodium hypochlorite for 10 min for berries and 2.5% for 3 min for leaves followed by three washes with sterile distilled water. They were then cut into 1-cm pieces and cultured on solid nutrient medium as explained by Krasnow and Murphy (2004) for callus production. This medium contained Gamborg 'B5' (1968) macronutrients, Murashige and Skoog 'MS' (1962) micronutrients and Morel (1970) vitamins supplemented with 20 g/L sucrose and 250 mg/L casein hydrolysate plus 0.2 mg/L kinetin and 0.1 mg/L NAA solidified with 7 g/L agar. Four explants were cultured in each 9 cm Petri dish and incubated at 24°C under a 16-h photoperiod with light intensity = 2250 lux supplied by 3 cool fluorescent lamps (Osram, 40 W each) at a distance of 30 cm.

In order to establish a cell suspension culture system, 1 g callus was separated from each sample and transferred into a 250-ml conical flask containing 50 ml of the same culture media as used for callus production but without agar and placed in a shaking incubator at 100 rpm under the same culture conditions. Suspension cultures were subcultured every 7 days by transferring 5 ml of the previous cultures into 50 ml fresh medium under aseptic conditions.

The stimulating effect of UV irradiation on resveratrol production by cells was evaluated by exposing suspension cultures to UV light ($\lambda = 254$ nm) 6 days after the third subculture when the cells were in the exponential growth stage. Treated suspension cultures were then kept for 24 h in the same incubation conditions before the amount of resveratrol in the cells was measured.

Extraction of resveratrol from the cells was carried out by vacuum filtration of suspension cultures, by placing 1 g of the cells into 20 ml absolute methanol, keeping at 4°C for 10 h, stirring on a magnetic stirrer for 30 min and finally centrifuging at 4000 rpm for 2 min. 20 μ l of supernatant from each sample was injected into an HPLC for the measurement of resveratrol using the same procedure as described for leaves and fruits in our previous report (Esna-Ashari *et al.* 2008) which was followed the analytical procedure of López *et al.* (2001) as a reference. Analyses were performed in a liquid chromatography with GBC (Australia) LC 1,150 pumps, software Winchrom V1.32, and 1,205 K photodiode array detector. Separation was carried out using an Exsil C₁₈ cartridge (250 × 4.6 mm ID, 4 μ m) and a guard cartridge. A 20 μ l injection loop has also been employed. The mobile phase consisted of acetic acid (A), methanol (B) and water (C). The composition was A:B:C (5:15:80, v/v/v) and the gradient program was as follows: 0.4 mL min⁻¹, 5 min. A:B:C (5:20:75), 0.5 mL min⁻¹, 30 min. A:B:C (5:45:50), 0.5 mL min⁻¹, 10 min. The signal response was monitored at 306 nm.

All experiments were conducted based on a complete randomized block design with three replications, the data analyzed using SAS software (version 9.1) and the means compared with the Duncan's multiple range test ($P < 0.05$).

RESULTS AND DISCUSSION

Callus was initiated from leaf and fruit explants of both grape cultivars 1 and 2 weeks after culture, respectively. Growth of the calli derived from leaf explants was faster as they became ready to subculture within 4 weeks, 6 weeks

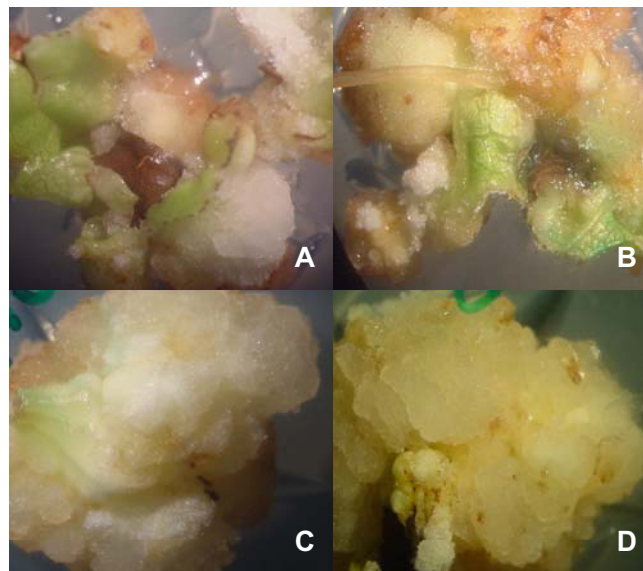


Fig. 1 Initiated callus from the leaf explant of 'Rajabi Sefid Shiraz' (A) and 'Keshmeshi Ghermez' (B) one week after culture, the leaf calli of 'Rajabi Sefid Shiraz' (C) and 'Keshmeshi Ghermez' (D) 4 weeks after culture and ready to subculture.

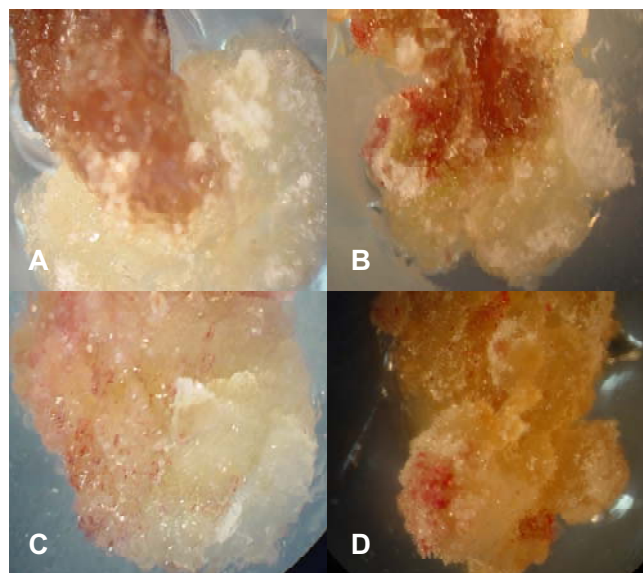


Fig. 2 Initiated callus from the fruit explant of 'Rajabi Sefid Shiraz' (A) and 'Keshmeshi Ghermez' (B) 2 weeks after culture, the fruit calli of 'Rajabi Sefid Shiraz' (C) and 'Keshmeshi Ghermez' (D) 6 weeks after culture and ready to subculture.

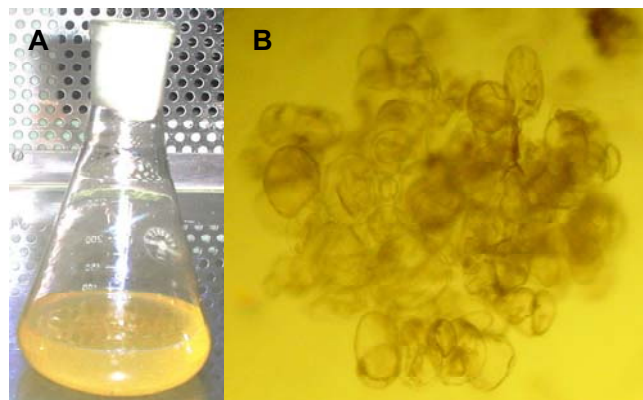


Fig. 3 Exponential growth stage of an established grape cell suspension culture (A) and a microscopic view of the cells in liquid medium (B).

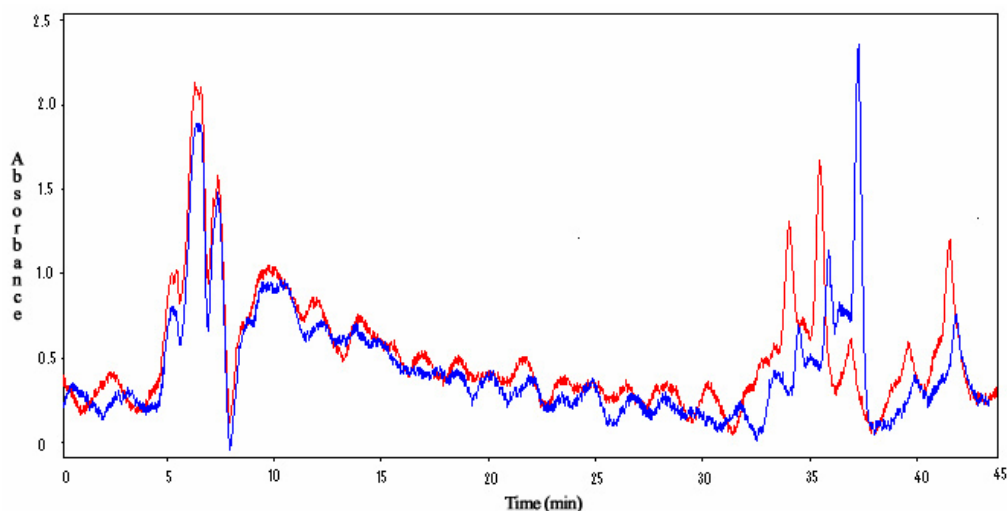


Fig. 4 A sample of chromatogram showing the peak of *trans*-resveratrol in grape extract (red line) and standard solution + extract (blue line) in cv. 'Rajabi Sefid Shiraz'.

for fruit explants (Figs. 1, 2). There was no significant difference between the two cultivars in terms of callus production from leaves or fruits. The calli derived from leaves and fruits were subcultured every 3 and 4 weeks, respectively to maintain the callus friable and suitable for suspension cultures, which were established after three subcultures. 6 days after the third subculture, cells were in the exponential growth stage when the color of liquid culture media appeared to be turbid confirming the highest number of cells in suspension cultures (Fig. 3). At this point, the cells were exposed to UV light.

The amount of resveratrol in organs and cells was determined through the liquid chromatography followed by producing analytical chromatograms. One of the chromatograms is shown in Fig. 4 as a sample. The results of resveratrol determination showed that there was a significant difference between leaves and fruits in the production of this compound in both cultivars: fruits produced more resveratrol than leaves (Table 1). The effect of UV light on the production of resveratrol in these organs is also shown in Table 1. Although the amount of resveratrol in UV-treated fruits and leaves of both cultivars increased, only leaves were significantly different from the controls. This is possibly due to the physical shape of the leaves, as they are flat and may receive more UV rays in comparison with the spherical-shaped berries. These findings, on the other hand, clarify that different organs of grapes may have different capacities of producing secondary metabolites. Similar results have also been reported by Ector *et al.* (1996) and Jeandet *et al.* (1995). These variations could possibly be associated with the different stilbene synthase enzyme activity levels in leaves and fruits of grapes. These, however, need further clarification especially at the molecular level. The stimulatory effect of UV light on the production of resveratrol in both organs of Iranian grape was clearly observed in this study. In the work of LeBlanc (2006), stilbenes accumulated in grape leaves and fruits when they were exposed to the UV light ($\lambda = 254$ nm) for 30 min. Douillet-Breuil *et al.* (1999) reported that the resveratrol-producing reaction of fruits to UV light was higher when they were younger and this gradually decreased when fruits ripened; leaves followed the same trend regardless of age. The authors stated that this might be due to the competition between the metabolic pathways of resveratrol and sugar or anthocyanin (red grapes) production during the ripening process.

In order to assess whether the individual cells of grape organs in suspension culture systems, with or without using an elicitor, are capable of producing resveratrol, the amount of this phenolic compound in UV-treated and non-treated cells derived from the calli of leaf and fruit explants of the

Table 1 Resveratrol production (mg/kg) in UV-treated and non-treated leaves and fruits of two Iranian grape cultivars.*

Sample	Cultivar	
	Rajabi Sefid Shiraz	Keshmeshi Ghermez
Fruit	3.35 a	1.62 a
Leaf	1.35 b	0.85 b
Fruit	3.35 a	1.62 a
Fruit (UV)	3.50 a	1.74 a
Leaf	1.35 b	0.85 b
Leaf (UV)	2.32 a	1.42 a

* The means in each column show significant differences according to Duncan's multiple range test ($P < 0.05$).

Table 2 The amount of resveratrol (mg/kg) in UV-treated and non-treated cells derived from the calli of leaf and fruit explants of two Iranian grape cultivars.*

Sample	Cultivar	
	Rajabi Sefid Shiraz	Keshmeshi Ghermez
Fruit cells	4.59 a	2.62 a
Leaf cells	3.62 b	1.08 b
Fruit cells	4.59 b	2.62 b
Fruit cells (UV)	7.73 a	5.22 a
Leaf cells	3.62 b	1.08 b
Leaf cells (UV)	5.69 a	2.26 a

* The means in each column show significant differences according to Duncan's multiple range test ($P < 0.05$).

Table 3 Comparison of resveratrol content (mg/kg) of UV-treated and non-treated organs and cells in two Iranian grape cultivars.*

Sample	Rajabi Sefid Shiraz		Keshmeshi Ghermez	
	Control	Treated	Control	Treated
Leaf	3.35 b	3.50 c	1.62 b	1.62 c
Fruit	1.35 c	2.32 d	0.85 c	1.42 c
Leaf cells	4.59 a	7.73 a	2.62 a	5.22 a
Fruit cells	3.62 b	5.69 b	1.08 c	2.26 b

* The means in each column show significant differences according to Duncan's multiple range test ($P < 0.05$).

two grape cultivars was also determined. The results showed that these cells could not only produce resveratrol in suspension cultures (Table 2), but also accumulate this compound even more when treated with UV light. There was a significant difference between cells derived from the calli of leaves and fruits of both cultivars in terms of resveratrol production. There have been substantial studies showing the stimulatory effect of UV light on stilbene synthase activity in grape cells resulting in the production of significantly more resveratrol in suspension cultures (Fritzscheier and Kindl 1981; Cantos *et al.* 2001; Versari *et al.* 2001; Jeandet *et al.* 2002; Krasnow and Murphy 2004).

Results of the present study on the Iranian grape cultivars are in agreement with the above groups' findings.

We finally analyzed the data to compare both intact leaves and fruits with their cells suspended in liquid culture media in terms of resveratrol production with or without UV light treatment (Table 3). Surprisingly, the results showed that freely suspended individual grape cells derived from highly differentiated organs like leaves and fruits could produce more resveratrol in suspension cultures. From a physiological point of view, when the cells are oriented in a highly differentiated and organized part of the plant, they can, as a whole, participate to generate any kind of secondary metabolite better than the individual single cells that are not connected to each other in liquid media. Molecular biological studies on the pathways of resveratrol synthesis in both organs and individual cells in suspension cultures are needed to clarify this. Considering the advantages of cell suspension culture systems for the production of secondary metabolites, we designed this study to see whether we could produce this valuable phenolic compound by Iranian grape cells in this system.

On the basis of our findings, we produced resveratrol from UV-treated treated cells by the use of a 2-l small lab-scale bioreactor successfully and the procedure is ready to fix larger bioreactors for the commercial mass production of this valuable compound.

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