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Production of *trans*-resveratrol in 'Cabernet Sauvignon' (*Vitis vinifera* L.) callus culture in response to ultraviolet-C irradiation

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Summary

In this study, the effects of ultraviolet (UV) irradiation time, incubation time and callus age were investigated for the induction of *trans*-resveratrol production in callus cultures of *Vitis vinifera* L. 'Cabernet Sauvignon'. Callus tissues were exposed to 254 nm UV light at 10 cm distance from the source for 10 and 15 min by opening covers of the petri dishes in sterile cabin. High Pressure Liquid Chromatography (HPLC) was used for the determination of *trans*-resveratrol production and concentrations were recorded at 0, 24, 48 and 72 hours after beginning of incubation. Separation by HPLC was achieved using a C18 column and a gradient elution with acetonitrile and water (from 10 to 85 % acetonitrile). The peak of *trans*-resveratrol was detected at 330 nm and identified from the retention time (12.5 min) *trans*-resveratrol standard. Determination coefficient for linearity (R^2), Limit of Detections (LOD), Limit of Quantification (LOQ) and relative standard deviation (RSD) values of the method were found as 0.9994, 0.12, 0.35 and 1.9, respectively. The highest *trans*-resveratrol concentration (62.66 $\mu g \cdot g^{-1}$ callus fresh weight) was determined in 48 hours of 12 days-old callus cultures irradiated for 10 minutes. Both 10 min and 15 min UV irradiation periods were found to be effective for the induction of *trans*-resveratrol production and thus callus cultures could be convenient for *trans*-resveratrol production.

K e y w o r d s : 'Cabernet Sauvignon' (*Vitis vinifera* L.), *trans*-resveratrol, callus culture, UV irradiation.

Introduction

Resveratrol (3-4'-5-trihydroxystilbene), is a secondary plant metabolite which has recently gained remarkable importance in the literature of medicine and pharmaceutics. It is stated in many studies that *trans*-resveratrol is a strong antioxidant reducing the risks of coronary heart disease as well as preventing the formation of cancer cells (Frankel *et al*. 1993, Koop 1998, Sárdi *et al.* 2000, Falchetti *et al*. 2001, Moriartry *et al*. 2001, Cui *et al.* 2002). The particular interest in this substance stems from a traditional medicine, "kojo-kon", derived from the roots of *Polygonum cuspidatum* plant which has been regarded as a public medicine in Japan and China for many years. This medicine has been used in the treatment of a range of diseases such as hypertension, arteriosclerosis and atherosclerosis, skin inflammation and allergy (Goldberg *et al.* 1996). Many of the species capable of producing resveratrol are wild species which are not used in agricultural production. The number of species that can directly be utilized in human nutrition is also very limited. Grapevine, mulberry, huckleberry, peanut and pistachio can be mentioned as the main representatives of these species. Compared to its counterparts, grapevine has a greater capacity of producing resveratrol and due to the widespread usage of its raw or processed products, it has been regarded as a valuable species and has been an important research topic. Resveratrol is a stilbene group phytoalexin which is a structural element in woody tissues of the grapevines such as the seeds, root and old branches while being synthesized in grain shells and leaves against biotic and abiotic stress.

The material required for obtaining secondary products is mostly achieved from the plants gathered from natural cultivation areas. Additionally, cultures of some plants are formed for this purpose. In both conditions which are named as "Traditional Methods", the amount of obtained pure substance varies according to the amount and quality of the vegetal material. In both cases, it is not possible to fulfill the production independent of the positive or negative ecological circumstances plants are surrounded with. Hence, as far as the achievement of secondary products is concerned, the purpose is to benefit from controlled conditions and the systems best utilising unit area.

This study aims at the determination of *trans*-resveratrol production capacity in 'Cabernet Sauvignon' (*Vitis vinifera* L.) by utilizing callus culture method and the elicitor effect of UV light. Within this framework, the effects of UV irradiation time, incubation time and callus age were investigated the induction of *trans*-resveratrol production in callus cultures of 'Cabernet Sauvignon'.

Material and Methods

C h e m i c a l s : All solvents for high pressure liquid chromatography (HPLC) was from Merck (Darmstadt, Germany). *Trans*-resveratrol (Cat. No. R 5010) was supplied by Sigma-Aldrich (St. Louis, MO).

P l a n t m a t e r i a l : *Vitis vinifera* L. 'Cabernet Sauvignon', was cultivated in the Viticultural Research Station of the Faculty of Agriculture, Ankara University.

E s t a b l i s h m e n t o f c a l l u s c u l t u r e : Leaf explants were used during the studies of callus culture. Explants were obtained from the grapes during the

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winter period and two-bud cuttings were utilized which were made of cuttings stored in polyethylene bags at +4 °C cold storage. The cuttings were planted in the greenhouse to the polyethylene bags containing sand, perlite and torf at a ratio of 1:1:1. The leaves were washed firstly in tap water and then in pure water. After being placed in a 20 % sodium hypochlorite solution with 0.01 % Tween 20 for 15 min, they were rinsed with sterilized pure water in 3 rounds of 5 min each and prepared for plantation. In terms of medium, Gamborg B-5 solid medium (Sigma G5893) was used (Gamborg *et al*. 1968). The pH value of the nutritional medium has been set to 5.7 by adding 3.2 $g \cdot l^{-1}$ ready-mixed medium in pure water. As a plant growth regulator, 1.0 µM BAP (6- benzylaminopurine) and 0.1 µM 2,4-D (2,4- dichlorophenoxy acetic acid) were added to reinforce callus development (Keller *et al*. 2000). The nutritional medium, after being supplied with saccarose (2 %) and agar (0.8 %), has undergone a sterilization process at autoclave for 20 min at 121 °C. Leaf pieces (1 cm^2), were planted in petri dishes of 100 x 200 mm with 30 ml medium inside. 15 petri dishes were prepared, each having 11 leaf explants. The calli were incubated in darkness and 25 °C, were sub-cultivated in two rounds having a 21 d-interval in between. After the second sub-cultivation, calli were transferred to fresh media and left to grown for 12 and 15 d until two different stages. These stages are specified as "callus age" in this study.

Elicitor treatment: The effect of short wave UV light was investigated in this study as an elicitor. Vilber-Lourmat T-15C UV-C lamp with 254 nm wave length was utilised as the light source. The UV light was used from a distance of 10 cm for 10 min or 15 min, respectively. For this purpose, the 12 and 15-d-old cultures were exposed to the light by removal of the petri dish caps in the sterilized cabin. The callus cultures were incubated at 25 °C in the dark for three different times of 24, 48 and 72 h. At the end of incubation, 1 g of the callus was wrapped in aluminium foil and stored at -80 °C till the analysis. Controls were taken the same way from 12 and 15 d-old cultures.

Trans-resveratrol extraction: *Trans*-resveratrol was extracted following a procedure established by Keller *et al*. (2000). The samples were homogenized (8000 rpm) with 10 ml of cold (-20 °C) acetone. After shaking for 30 min, the homogenate was centrifuged at 3000 x *g* for 10 min, and the supernatant was retained. The extraction was repeated on the pellet once with acetone and once with acetone: methanol (1:1 v/v). All extracts were combined centrifuged again and remaining cell debris was frozen out (-20 °C) overnight. After decanting, the solvent was evaporated under a stream of nitrogen in a 40 °C water bath to less than 1 ml. The volume was adjusted with methanol to 2 ml and sample was filtered through a 0.45 µm syringe filter for HPLC analysis.

HPLC analysis: HPLC analyses were carried out using a method established by Jeandet *et al*. (1997). 5 µl of each sample was injected for HPLC analyses. Nucleodur 100-5 C-18 column, reversed phase column (250 x 4.6 mm) preceded by a guard column (CC 8/4 Nucleodur 100-5 C-18), SSI Lab Alliance Essence HPLC Instrument and UV-VIS detector were used in this study. The flow rate was 1 $ml \cdot min^{-1}$. *Trans*-resveratrol was eluted from HPLC C-18 column with gradient comprising acetonitrile (solvent A) and water (solvent B). Solvents were delivered according to the following program: linear gradient elution from 10 % A and 90 % B to 85 % A and 15 % B within 18 min; 85 % A and 15 % B for 5 min; linear gradient elution from 85 % A and 15 % B to 10 % A and 90 % B within 7 min. This was followed by a 5 min equilibrium period with 10 % A and 90 % B prior to injection of the next sample using gradient elution at a flow rate of 1.0 $ml \cdot min^{-1}$.

Identification and quantification of *trans*-resveratrol: Identification of *trans*-resveratrol was achieved through comparison with known standards (Jeandet *et al*. 1997). The peak of *trans*-resveratrol was detected at 330 nm and identified from the retention time (12.5 min of a *trans*-resveratrol standard). *Trans*-resveratrol concentrations were expressed as $\mu g \cdot g^{-1}$ fw (µg resveratrol per 1 g callus fresh weight, fw). Determination coefficient for linearity (R^2), Limit of Detections (LOD), Limit of Quantification (LOQ) and relative standard deviation (RSD) values of the method were found as 0.9994, 0.12, 0.35 and 1.9, respectively.

Data analysis: Experiments and analyses were repeated in three rounds. In both of the callus ages (12 and 15-d-old). The effects of UV irradiation time and incubation time on *trans*-resveratrol concentration were examined. The obtained measurement values were analysed according to "Factorial Analysis of Variance Method" (Winer *et al*. 1991). In order to determine the differences, a multiple comparison test of "Least Significant. Difference (LSD) Test" was used. Differences were analysed at a significance level of 1 % ($p < 0.01$). STATISTICA (ver: 6.0) and SPSS (ver: 13.0) programs were used for statistical analyses.

Results

The effects of UV irradiation time and incubation time on *trans*-resveratrol concentration: After a UV irradiation of 10 min on 12 d-old callus cultures, the *trans*-resveratrol concentration which was measured as 1.49 $\mu g \cdot g^{-1}$ fw after 24 h has increased almost 42 times as much and reached 62.66 $\mu g \cdot g^{-1}$ fw after 48 h. Measurement after 72 h indicated a dramatic decrease in the *trans*-resveratrol concentration to 28.27 $\mu g \cdot g^{-1}$ fw (Table). At a UV irradiation of 15 min the *trans*-resveratrol concentration was 1.21 $\mu g \cdot g^{-1}$ fw after 24 h and the value increased 48 times to 58.47 $\mu g \cdot g^{-1}$ fw and decreased to 53.81 $\mu g \cdot g^{-1}$ fw after 72 h (Table). When the results of both above mentioned UV irradiations on 12 d-old callus tissues are compared with those of the control group (0.85 $\mu g \cdot g^{-1}$ fw), the value of *trans*-resveratrol concentration was significantly higher than that of the control group. When 10 and 15 min UV irradiations were compared, the *trans*-resveratrol concentration (62.66 $\mu g \cdot g^{-1}$ fw) reached after a 10 min UV irradiation after 48 h was remarkably higher than the values obtained at the end of the other two exposure times. On the other hand, no significant difference was obtained be-

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The effects of UV irradiation and incubation times on *trans*-resveratrol concentration in 12- and 15-d-old calli of 'Cabernet Sauvignon'

	Irradiation time (min)	Incubation time (h)			
		24	48	72	Mean
		Mean ± St. Err.	Mean ± St. Err.	Mean ± St. Err.	Mean ± St. Err.
12-d-old calli	10	A 1.49 ± 0.09 c	A 62.66* ± 0.40 a	B 28.27* ± 0.02 b	30.80 ± 11.19
	15	A 1.21 ± 0.01 c	B 58.47* ± 0.11 a	A 53.81* ± 0.07 b	37.83 ± 11.61
	Mean	1.35 ± 0.08	60.56 ± 1.22	41.04 ± 7.37	
	Control mean	0.85 ± 0.04	LSD = 0.77		
15-d-old calli	10	B 4.45* ± 0.03 b	B 18.12* ± 0.10 a	B 3.94* ± 0.10 b	8.83 ± 2.93
	15	A 17.46* ± 0.34 b	A 28.41* ± 0.02 a	A 14.20* ± 0.01 c	20.02 ± 2.72
	Mean	10.05 ± 3.75	23.26 ± 2.97	9.07 ± 2.96	
	Control mean	0.45 ± 0.01	LSD = 0.67		

The difference among the means in the same row with different small letters is significant ($p < 0.01$).
The difference among the means in the same column with different capital letters is significant ($p < 0.01$).
*The difference from the control group is statistically significant ($p < 0.01$).

tween the 10 and 15 min UV irradiations after 24 h while a 15 min irradiation was found to be more effective than 10 min after 72 h. Having evaluated the UV irradiations (for *trans*-resveratrol production) after 15 d of the callus cultures with respect to irradiation time the *trans*-resveratrol concentration which was 0.45 $\mu g \cdot g^{-1}$ fw in the control group, has changed to 4.45 $\mu g \cdot g^{-1}$ fw, 18.12 $\mu g \cdot g^{-1}$ fw and 3.94 $\mu g \cdot g^{-1}$ fw after 24, 48 and 72 h, respectively, as a result of 10 min UV radiation. In case of 15 min UV irraditation, *trans*-resveratrol concentration was 17.46 $\mu g \cdot g^{-1}$ fw, 28.41 $\mu g \cdot g^{-1}$ fw and 14.20 $\mu g \cdot g^{-1}$ fw after 24, 48 and 72 h, respectively (Table). When the 10 and 15 min UV irradiations in this group are compared against the measurements after 3 h, 15 min UV irradiation lead to greater amounts of *trans*-resveratrol than the 10 min irradiations. Hence, 15 min UV irradiation was found to be more effective for high amount of *trans*-resveratrol production in 'Cabernet Sauvignon'.

T h e e f f e c t o f c a l l u s a g e s o n *t r a n s*-r e s v e r a t r o l p r o d u c t i o n: On the basis of the measurements after 48 h with the highest *trans*-resveratrol concentrations, the effect of callus age on *trans*-resveratrol production was examined. Ten min UV irradiation was found to be more effective for the 12-d-old calli and the highest *trans*-resveratrol concentration value (62.66 $\mu g \cdot g^{-1}$ fw) in 'Cabernet Sauvignon' was achieved. For the 15 d-old calli, 15 min UV radiation was proven to be more effective than 10 min irradiation. The *trans*-resveratrol concentration values of the 15 d-old calli after 48 h were measured respectively as 28.41 $\mu g \cdot g^{-1}$ fw and 18.12 $\mu g \cdot g^{-1}$ fw, in 15 min and 10 min irradiations. In general, *trans*-resveratrol production was more successful with 12 d-old calli than with 15 d-old counterparts.

Discussion

Following the 10 and 15 min UV irradiation experiments, an increase was observed after 24 h and the *trans*-resveratrol concentrations reached their maximum values after 48 h. *Trans*-resveratrol has decreased after 72 h. Douillet-Breuil *et al*. (1999) found out that *trans*-resveratrol concentration in grapevine species increased in two stages - the first after 20 h and the second after 40 h reaching the maximum values in the second incubation time.

On the other hand, the decrease in concentration after 72 h is an expected outcome. This case, as also stated by Keller *et al*. (2000), can be explained by the decrease in *trans*-resveratrol synthesis accompanied by the aging of calli. In addition, the short-term permanency of secondary products can be attributed to the breakdown of tissues by enzymes after a certain stage of incubation (Charlwood *et al*. 1990). In the control calli, *trans*-resveratrol concentrations were 0.85 $\mu g \cdot g^{-1}$ fw and 0.45 $\mu g \cdot g^{-1}$ fw in the 12- and 15 d-old cultures. The low amount of *trans*-resveratrol concentration in the control calli can result from the possible role of *in vitro* conditions as stress factors for the cells. Keller *et al*. (2000) also stated that the decrease in medium sugar content during cultivation conditions might be a stress factor.

Considering the highest value of *trans*-resveratrol concentration in controls and experiments, it was determined that UV irraditation in 'Cabernet Sauvignon' stimulated the *trans*-resveratrol production by 28 times. Five main outcomes have been identified in current findings and former studies:

within this work: (1) UV irradiation lead to production of high amounts of *trans*-resveratrol in the callus tissues of 'Cabernet Sauvignon' (2) UV light has proven to be an effective elicitor for callus (3) 48 h was determined as the incubation time displaying the highest *trans*-resveratrol concentration in both UV irradiation times and both callus ages (4) It was identified that the "quality" and "age" of the calli to be exposed to the elicitor are important determinants for the success of the experiment (5) It was concluded that callus cultures can be used as model systems for the stimulation and determination of *trans*-resveratrol production in grapevines.

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