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PRODUCTION OF *TRANS*-RESVERATROL IN CALLUS TISSUE OF ÖKÜZGÖZÜ (*VITIS VINIFERA* L.) IN RESPONSE TO ULTRAVIOLET-C IRRADIATION

N. Keskin and B. Kunter*

Yüzüncü Yıl University, Faculty of Agriculture, Department of Horticulture, 65080 Van, Turkey
*Ankara University, Faculty of Agriculture, Department of Horticulture, 06110 Ankara, Turkey
Corresponding author E-mail: nurhan_keskin@yahoo.com

ABSTRACT

In this study, the effects of ultraviolet (UV) irradiation time, incubation time and callus age were investigated for *trans*-resveratrol induction in callus cultures of *Vitis vinifera* L. cv. Öküzgözü. Callus tissues were exposed to 254 nm UV light at 10 cm distance from the source for 10 and 15 minutes by opening covers of the petri dishes in a sterile cabinet. High Pressure Liquid Chromatography was used for determining *trans*-resveratrol production and concentrations were recorded at 0, 24, 48 and 72 hours after beginning of incubation. The highest *trans*-resveratrol concentration (62.23 µg/g callus fresh weight) was determined at 48 hours of 12 -day-old callus cultures irradiated for 15 min. 15 min UV irradiation period was found to be effective for induction of *trans*-resveratrol production and thus callus cultures could be convenient for *trans*-resveratrol production.

Key words: Öküzgözü (*Vitis vinifera* L. cv.), *trans*-resveratrol, callus culture, UV irradiation

INTRODUCTION

Plants can synthesize particular molecules called secondary metabolites or secondary productions which are specific to species and differ from the primary productions such as proteins and lipids. These particular molecules are not directly correlated with growth and development of the plants. Secondary metabolites have been important for humans who use plants for various purposes for centuries as well as they have direct importance to plants. Tastes and aroma of foodstuffs obtained from the plants are caused by these productions. Caffeine in the coffee and nicotine in tobacco can be given as the most common examples for these. Another and most important field of use for these secondary metabolites is to use them for therapy for some diseases such as cancer, corronary heart diseases etc. Nowadays, secondary metabolites are valuable and important raw materials for many areas such as cosmetics, pharmaceuticals, agricultural chemistry and additives. Considering that 25% of the chemicals (alkaloids, steroids etc.) are originated from the plants, it is obvious that natural vegetal resources have high economic values (Bourgaud *et al*., 2001; Ramachandra and Ravishankar 2002). Traditional methods are being used to obtain secondary metabolites from the plants. However, amount of the pure matters obtain change with the quantity and quality of the vegetal material. For this reason, secondary metabolites are produced under controlled conditions. Many of the species capable of producing secondary metabolites are wild species which are not used in agricultural production. Grapevine has widespread usage of its fresh or processed product. Thus, it has been regarded as a valuable species and has been an impartont reserach topic. Grapevine species are able to produce the organic compounds grouped in stilbene as a secondary metabolite.

Many stilbene compounds (*trans*-resveratrol, piceattannol, piceid and pterostilbene) which were clarified the chemical structure are essential for human nutrition and health, thus grapevine species have an important role in bioengineering studies.

In the present study, the effect of UV irradiation on *in vitro trans*-resveratrol production capacity of Öküzgözü grape cultivar was determined.

MATERIALS AND METHODS

Plant material: *Vitis vinifera* L. cv. Öküzgözü, is an indigenous, high quality red wine variety originally grown in Elazığ, Malatya, Diyarbakır and Adıyaman provinces of Turkey. Dormant cuttings of Öküzgözü cv. were obtained from the gene bank of Viticultural Research Station of Faculty of Agriculture, Ankara University.

Establishment of callus culture: Explants were obtained from dormant cuttings grown in a greenhouse during winter. The leaves were first washed in tap water and then in distilled 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 three times for 5 min each and prepared for plantation. Gamborg B-5 basic solid nutritional medium (Sigma G5893) was used (Gamborg *et al*., 1968). The pH value of the nutritional medium was set to 5.7 by adding 3.2 g/L ready-mixed nutritional 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). After being supplied with sucrose (2%) and agar (0.8%), the nutritional medium was autoclaved for 20 min at 121°C for sterilization.

Leaf pieces (1 cm^2) were planted in the petri dishes of 100x200 mm filled with 30 mL medium. Fifteen petri dishes each having eleven leaf explants were used. The calli incubated in dark at 25°C were sub-cultivated twice with 21 day-interval. After the second sub-cultivation, calli were transferred to fresh media and left for growth for 12 and 15 days for reaching two different ages.

Elicitor treatment: The effect of shortwave UV light as an elicitor was investigated in this study. Vilber-Lourmat T-15C UV-C lamp with 254 nm wave length was utilised as the light source. The UV light was applied from a distance of 10 cm for 10- and 15 minutes onto the 12 and 15-day-old cultures by the removal of the petri dishes in the sterile cabin.

Callus cultures were incubated at 25°C in dark for 24, 48 and 72 hours. At the end of incubation, calli were weighed as 1g, wrapped in aluminum foil and stored at -80^{o}C until the analyses. Controls were taken from 12 and 15-day-old cultures as 1 g from each and samples were stored in aluminium foil at -80^{o}C till the time of analysis.

***Trans*-resveratrol extraction:** *Trans*-resveratrol was extracted following a procedure established by Keller *et al*. (2000). The samples were standardized (8000 rpm) with 10 mL of cold (-20^{0}C) acetone. After shaking for 30 minutes, the homogenate was centrifuged at 3000x*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 were frozen out (-20 ^{0}C) overnight. After decanting, the solvent were 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 min^{-1}. T*rans*-resveratrol was eluted from HPLC C-18 column with gradient comprising solvents acetonitrile (A) and water (B). Solvents were delivered according 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 equilibration period with 10% A and 90% B prior to injection of the next sample using gradient elution at a flow rate of 1.0 $mL.min^{-1}$.

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 *trans*-resveratrol standard. *Trans*-resveratrol concentrations were expressed as µg resveratrol/1 g callus fresh weight (µg/g fw).

Data analysis: Experiments were conducted with three replication. In both of the callus ages (12 and 15-days-old), the effects of UV irradiation time and incubation time on *trans*-resveratrol concentration were examined. The obtained values were analysed according to "Factorial Analysis of Variance (Two- way Factorial ANOVA)" (Winer *et al.,* 1991). In order to determine the differences, a multivariate analysis technique of " Least Significant Difference (LSD) Test" was used. Differences were analyzed at a significance level of 1% ($p<0.01$). STATISTICA and SPSS applications were used for statistical analyses.

RESULTS AND DISCUSSION

In 12 and 15-days-old callus cultures, 10 and 15 min UV irradiation showed similar effects on resveratrol accumulation. It was observed a small increment in the first 24 hours observed with the highest accumulation after 48th hour and dramatic decrease at the 72nd hour (Table 1).

Resveratrol was 8-10 times higher in the 48th hour in the 12-day-old cultures than in the first 24 hours after UV application. The highest resveratrol accumulation was 62.23 µg/g fw in 15 minute application after 48th hour (Table 1). 15-days-old callus cultures gave the highest amount (41.98 µg/g fw) in the 10 minute application at the 48th hour (Table 1).

In this research, control calluses were shown to produce *trans*-resveratrol concentrations between 1.94-2.37 µg/g fw. When the highest *trans*-resveratrol concentration (62.23 µg/g fw) obtained from the applications was considered, it was exhibited that UV light treatment increased *trans*-resveratrol accumulation 26-fold in Öküzgözü. UV light was found to be quite effective on callus cultures. This finding was in agreement with the other studies (Barlass *et al*., 1987, Creasy and Coffee 1988; Sbaghi *et al*., 1995; Bais *et al.,* 2000; Versari *et al.,* 2001) who reported that UV light consistently produces more *trans*-resveratrol compared to the other elicitors. However, the efficiency of UV light

for trans-resveratrol production depends on the time of exposure. Like the studies which investigated the effects of UV light exposure time on induction of *trans*-resveratrol and other stilbene compounds (Langcake and Pryce 1977; Barlass *et al*., 1987; Sbaghi *et al*., 1995; Douillet-Breuil *et al*., 1999; Pezet *et al*., 2003), the success of exposure time in this research changed with callus age and incubation time. Keskin and Kunter (2007) found that callus quality and age were effective on *trans*-resveratrol production of Erciş grape cultivar, and generally 12-day-old cultures were more productive than 15 day- old ones. Douillet-Breuil *et al*. (1999) reported that the 10 and 15 minute UV light application caused an increase in *trans*-resveratrol accumulation in the first 24 hours, peaked at the 48th hour and showed a decrease at the 72th hours. Our findings support their results.

We recommend that in order to obtain high amount of resveratrol accumulation, callus cultures should be at least 12-days-old, exposed to UV light for 15 minutes and incubated for 48 hours. Consequently, it is suggested that callus cultures is used as model systems for induction and determination of *trans*-resveratrol production in grapevines. UV light leads to high accumulation of *trans*-resveratrol in callus cultures, and it is used as an effective elicitor depending on the quality and age of callus cultures.

Table 1. The effects of UV- C irradiation and incubation times on *trans*-resveratrol concentration in 12 and 15-d-old callus of Öküzgözü (*Vitis vinifera* L.)

	Irradiation Time (min)	Incubation Time (h) 24 Mean ± SE.	48 Mean ± SE.	72 Mean ± SE.	Mean Mean ± SE.
12-day old calli	10	B 5.54* ± 0.005 b	B 40.06* ± 0.05 a	B 2.54 ± 0.02 c	16.04 ± 7.61
	15	A 6.58* ± 0.070 c	A 62.23* ± 0.10 a	A 10.29* ± 0.10 b	26.36± 11.36
	Mean	6.06 ± 0.30	51.14 ± 6.40	6.41 ± 2.23	
	Control mean : 2.37 ± 0.02		LSD = 0.30		
15-day old calli	10	B 8.23* ± 0.10 b	A 41.98* ± 0.10 a	B 2.94* ± 0.02 c	17.71 ± 7.73
	15	A 19.86* ± 0.02 c	B 22.79* ± 0.09 a	A 20.28* ± 0.01 b	20.97 ± 0.57
	Mean	14.04 ± 3.35	32.38 ± 5.54	11.61 ± 5.00	
	Control mean : 1.94 ± 0.04		= 0.32		

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 ($p<0.01$).

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