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The Effects of Callus Age, UV Irradiation and Incubation Time on t*rans*-Resveratrol Production in Grapevine Callus Culture*

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Abstract: In this study, the effects of callus age, ultraviolet (UV) irradiation and incubation time were investigated for the induction of *trans*-resveratrol production in callus cultures of Kalecik karası (Clone 12) *Vitis vinifera* L. grape cultivar. Part of leaves (~1 cm^2) were taken from one year old grapevines grown in greenhouse and then were cultured in Gamborg B-5 media including 2% saccarose, 0.8% agar, 1.0 µM BAP (6-benzylaminopurine) and 0.1 µM 2, 4-D (2, 4-dichlorophenoxy-acetic acid). After the second subculture, 12 and 15 days old callus tissues were exposed to 254 nm UV light at 10 cm distance from the source for 10 and 15 min. *Trans*-resveratrol was measured at 0, 24, 48 and 72 hours of incubation by using High Pressure Liquid Chromatography (HPLC). *Trans*-resveratrol concentration of control callus ranged from 0.56 to 0.96 µg g fw^{-1}. The highest *trans*-resveratrol production was obtained from 12 and 15 days-old callus irradiated for 10 min. at the 48th hours of incubation (2.42 µg g fw^{-1}). Considering the highest value of *trans*-resveratrol concentration in controls and experiments, it was determined that UV irraditation in Kalecik karası elicitated the *trans*-resveratrol production by 2.5 times.

Key Words: Kalecik karası (*Vitis vinifera* L. cv.), stilbene, trans-resveratrol, callus culture, UV irradiation.

Asma Kallus Kültürlerinde *trans*-Resveratrol Üretiminin Uyarılması Üzerine Kallus Yaşı, UV Işını Uygulama ve İnkübasyon Sürelerinin Etkisi

Öz: Bu çalışmada, Kalecik karası Klon 12 (*Vitis vinifera* L) üzüm çeşidine ait kallus kültürlerinde, *trans*-resveratrol üretiminin uyarılması üzerine kallus yaşı, ultraviyole (UV) ışını uygulama ve inkübasyon süresinin etkisi incelenmiştir. Kallus eldesi için serada yetişen bir yaşlı asmalardan alınan yaprak parçaları (~1 cm^2), %2 sakkaroz, % 0.8 agar, 1.0 µM BAP (6-benzilaminopürin) ve 0.1 µM 2, 4-D (2, 4- diklorofenosi-asetik asit) eklenmiş Gamborg B-5 ortamında kültüre alınmıştır. İkinci alt kültürden sonra, 12 ve 15 gün yaşlı kalluslara, 10 cm uzaklıktan 10 ve 15 dakika süreyle 254 nm dalga boyuna sahip UV ışını uygulanmıştır.*Trans*-resveratrol üretiminin belirlenmesi amacıyla, Yüksek Basınçlı Sıvı Kromotografisi (HPLC) yönteminden yararlanılmış, ölçümler 0., 24., 48. ve 72. saatlerde yapılmıştır. *Trans*-resveratrol konsantrasyonu kontrol kalluslarında, 0.56-0.96 µg/g fw arasında değişmiştir. En yüksek *trans*-resveratrol üretimi, 10 dakika UV ışını uygulaması yapılmış 12 ve 15 gün yaşlı kalluslarda 48. saat ölçümlerinde elde edilmiştir (2.42 µg/g fw). Kontrol ve uygulamalarda elde edilen en yüksek *trans*-resveratrol derişimleri dikkate alındığında UV ışını uygulamasının Kalecik Karası'nda *trans*-resveratrol birikimini uyardığı ve yaklaşık 2.5 kat artış sağladığı belirlenmiştir.

Anahtar Kelimeler: Kalecik karası *(Vitis vinifera* L.), stilben, *trans*-resveratrol, kallus kültürü, UV ışını.

Introduction

Primary compounds produced by plant during the living circulation are important for living of all ecosystems. In addition, they are inevitable for human such as nutrition and housing. Moreover, different from primarily molecules, plants are able to synthesizing special molecules. These molecules are called as secondary compounds. The synthesizing of secondary compounds depends on plant exposed to biotic or abiotic stress factors. These compounds are generally playing an important role such as adaptation of the plants to environmental conditions and regulation of the relationships between ecosystems and plant health. Moreover, these compounds have direct effects for plants as well as indirect effects for human beings using these plants. Because of having pharmaceutics effects, plants and indirectly secondary compounds have been used for positive and negative aims for a long time. Nowadays, these products are valuable raw material for pharmaceutics, cosmetic, agricultural chemicals, and food industry. Resveratrol, one of these compounds, is a specific stilbene compound of grapevine. It is emphasized that resveratrol has the

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antioxidant, anti-tumor and anti-mutagen effects as well as decreasing the coroner heart diseases and preventing the formation of carcinoma cells in the medical and pharmacological literature (Briviba et al. 2002, Soleas et al. 2002). As other secondary metabolites, resveratrol is produced by related to the effects of biotic and abiotic elicitors as a result of stress mechanisms (Langcake and Pryce 1976, Adrian et al. 1996, Bonomelli et al. 2004). The quality and quantity of pure material in the grape berries and tissues in natural conditions are varied by the ecological conditions and growth levels of plant. Thus, it is aimed to use plant tissue culture techniques for continuous production and having stabile quality as well as solving the encountered problems in the extraction of secondary metabolites. Moreover, *in vitro* techniques have several advantages to increase productivity in the production of secondary metabolites. Thus, especially callus and cell suspension cultures are attractive (Lila 2005, Zamboni et al. 2006).

In this study, production possibility of resveratrol as a raw material in grapevine calli and tissues are studied. For this aim, callus cultures of Kalecik karası (Clone 12) which is one of the attractive grapevine cultivars for red wine in Turkey was selected as grapevine cultivar.

Material and Method

Chemicals: All solvents used for HPLC were of analytical grade. Methanol used for HPLC was purchased from Fisher (USA), the standard of *trans*-resveratrol was form Sigma (USA).

Plant material: Callus tissues obtained from Kalecik karası (Clone 12) grapevine cultivar were used in the study. The plantation of Kalecik karası is located in Viticultural Research Station of Faculty of Agriculture, Ankara University.

Tissue culture experiments: For callus culture experiments, leaf explants were obtained from the mid part of the shoots of potted one year old grapevines grown in greenhouse. Gamborg B-5 (Gamborg et al. 1968) basic solid medium (Sigma G5893) was used as nutritional medium. The pH value of the nutritional medium has been set to 5.7 by adding 3.2 g l^{-1} 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). 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 the petri dishes of 100x200 mm with 30 ml medium 15 petri dishes were formed, each having 11 leaf explants. The calli incubated in dark and 25°C were sub-cultivated in two times having a 21 day-intervals. After the second sub-cultivation, calli were transferred to fresh media and left to growth for 12 and 15 days for reaching two different stages. These stages are called as “callus age” in this study.

Elicitor treatment: The short wave UV light was used as elicitor. Vilber-Lourmat T-15C UV-C lamp with 254 nm wave length was utilized as the light source. The UV light was given from a distance of 10 cm for 10 and 15 minutes duration. The application of UV radiation was realized with opening covers of petri dishes in the sterile cabin. Following the application, callus cultures were exposed to three incubation times as 24, 48 and 72 hours at 25 °C in dark conditions. One g sample was taken from control and application callus at the 12^{th} and 15^{th} days of culture, wrapped in aluminum foil and stored at -80°C till the analysis.

HPLC (High Performance Liquid Chromatography): *Trans*-resveratrol level was detected by using HPLC system. Name of the system is SSI LabAlliance Esence HPLC Workstation and the system has Phenomenex/Luna guard colon (5 µm, 12.5 x 4.6 mm, ID), Phenomenex/Luna C18 colon (5 µm, 250 x 4.6 mm, ID) and UV-VIS detector. *Trans*-resveratrol was extracted following a procedure established by Keller et al. (2000). HPLC analyses were carried out by using a method established and identification of *trans*-resveratrol was achieved comparison with known standards by Jeandet et al. (1997). The peak of *trans*-resveratrol was detected at 330 nm and identified from the retention time (12.5 min) *trans*-resveratrol standard. *Trans*-resveratrol concentrations were expressed as µg g fw^{-1} (µg resveratrol in 1 g callus fresh weight, fw).

Data analysis: The data obtained from three replications were analyzed according to “Factorial ANOVA” (Winer et al. 1991). After ANOVA, Least Significant Difference (LSD) which is multiple comparison test was used to determine differences between means. $P<0.01$ was indicated as a significant level for all statistical comparisons. STATISTICA (ver: 6.0) and SPSS (ver: 13.0) programs were used for statistical analyses.

Results

According to the ANOVA results, “UV radiation x incubation time” interaction was significant ($P<0.05$). Thus, the effects of factors for resveratrol were evaluated as following;

1) The comparison of incubation times in the same UV irradiation,

2) The comparison of UV irradiation of in the same incubation times.

After a UV irradiation of 10 min on 12-day-old callus cultures, the *trans*-resveratrol concentration which was measured as 1.82 µg g fw^{-1} at the 24^{th} hour, reached to the maximum values (2.42µgg fw^{-1}) at the 48^{th} hour. The *trans*-resveratrol concentration (1.59 µg g fw^{-1}) was decreased in the latest incubation time (at the 72^{nd} hour).

In the case of a UV irradiation of 15 min, while the *trans*-resveratrol concentration was 1.95 µg g fw^{-1} at 24^{th} hour, the value reached to 2.16 µg g fw^{-1} and decreased to 1.55 µg g fw^{-1} at the 72^{nd} hour (Table 1). When the results of both above mentioned UV irradiations on 12-day-old callus tissues were compared with those of the control group (0.96 µg g fw^{-1}), it was seen in all treatments that the value of *trans*-resveratrol concentration was significantly higher than that of the control group.

When 10 and 15 min UV irradiations were compared; UV irradiation for 10 min was found as more effective than 15 min. It was determined that the *trans*-resveratrol concentration (2.42 µg g fw^{-1}) at the end of 48^{th} hour incubation time and 10 min UV irradiation was higher than the values obtained at the end of the other two exposure times. On the other hand, no significant difference was obtained between the 10 and 15 min UV irradiations at the 24^{th} and 72^{nd} hour.

Having evaluated the UV irradiations at the 15-day-old of the callus cultures -for the stimulation of *trans*-resveratrol concentration-with respect to irradiation time; the *trans*-resveratrol concentration which was measured as 0.56 µg g fw^{-1} in the control group, changed to 1.73 µg g fw^{-1}, 2.42 µg g fw^{-1} and 1.60 µg g fw^{-1} at the 24^{th}, 48^{th} and 72^{nd} hours, respectively, as a result of 10 min UV radiation. In case of 15 min UV irradiation, *trans*-resveratrol concentration was measured as 1.50 µg g fw^{-1}, 1.89 µg g fw^{-1} and 1.29 µg g fw^{-1} at the 24^{th}, 48^{th} and 72^{nd} hours, respectively (Table 1).

As compared 10 and 15 min UV irradiations, there was found significant difference between these times. Thus, 10 min UV irradiations was considered as more effective for *trans*-resveratrol production in Kalecik karası cultivar.

Because there was a significant interaction between two factors, the comparisons were performed in each UV irradiation and incubation time to offer the most favorable callus age. 48^{th} hour obtained the highest resveratrol based on the comparisons.

When the resveratrol production was considered, there was no significant difference between 12 and 15 -day-old calli exposed to 10 min UV irradiation and resveratrol concentration was measured as 2.42 µg g fw^{-1} for both callus ages. However, for 15 min UV irradiation, 12 -day-old calli were proved higher performance as compared to 15 day-old calli with regard to resveratrol accumulation (Table 1).

Discussion

It was determined that resveratrol concentration of control group ranged from 0.56 to 0.96 µg g fw^{-1} in the study. Following the 10 and 15 min UV irradiation, an increase was observed at the 24^{th} hour and the *trans*-resveratrol concentrations reached to their maximum values at the 48^{th} hour and then decreased by the 72^{nd} hour.

When the highest resveratrol concentration values obtained from the application and control groups were considered, it was detected that UV irradiation leaded to about two times increase of resveratrol accumulation. According to this result, it should be noted that UV irradiation can be effective application to increase resveratrol accumulation in calli cultures. On the other hand, because of the consistency of resveratrol concentrations obtained from both callus ages, it can be stressed that callus age to apply elicitor did not have significant effect on increasing resveratrol production in Kalecik karası grape cultivar. This finding does not agree with that of Keskin and Kunter (2007). They indicated that both age and quality of callus were effective for resveratrol production and generally resveratrol accumulation in 12 day-old calli was higher than that of 15 day-old calli in Erciş grape cultivar.

Nowadays it is observed that resveratrol is a considerable quality criterion for grape cultivars. In addition, the aim of almost every sector is to determine the factors to cause high resveratrol production. In according to this, it was emphasized that genotypes have different resveratrol production capacities as application of elicitor (Adrian et al. 1996, Sárdi et al. 2000). However, this cannot be independently explained due to the effects on genotypes by other factors. Adrian et al. (1996) explained importance of genotypes as "resveratrol production was genetically controlled whatever elicitor to be applied".

Nevertheless, *in vitro* researches are more convenient to investigate the effect of genotypes; difference between same genotypes cannot be

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

	Irradiation Time (min)	Incubation Time (h)			
		24	48	72	Mean
		Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE
12-day old calli	10	A 1.82* ± 0.02 b	A 2.42* ± 0.04 a	A 1.59* ± 0.02 c	1.94 ± 0.15
	15	A 1.95* ± 0.06 b	B 2.16* ± 0.020 a	A 1.55* ± 0.03 c	1.88 ± 0.11
	Mean	1.88 ± 0.046	2.29 ± 0.07	1.57 ± 0.01	
	Control mean :	0.96 ± 0.05	LSD = 0.14		
15-day old calli	10	A 1.73* ± 0.03 b	A 2.42* ± 0.10 a	A 1.60* ± 0.10 b	1.91 ± 0.16
	15	B 1.50* ± 0.10 ab	B 1.89* ± 0.09 a	A 1.29* ± 0.09 b	1.56 ± 0.11
	Mean	1.62 ± 0.13	2.15 ± 0.16	1.45 ± 0.06	
	Control mean :	0.56 ± 0.01	LSD = 0.40		

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).

blocked under various stress factors in a vineyard. The apparent capacity of a genotype may be exhibited different concentration value by effecting of determined factor as well as another factor. However, this takes part in the similar group as compared to other genotypes.

In our study, Kalecik karası showed low resveratrol accumulation. Anlı et al. (2006) compared Kalecik karası wine with that of native and foreign cultivars (Sauvignon, Öküzgözü, Boğazkere, Syrah and Pinot noir) in point of resveratrol concentration and defined this wine as “low concentration”.

This study is the first and plays an important role about investigation of the relationships between grape cultivar and resveratrol concentration exception of commercial grape productions. It is suggested that resveratrol production capacity of native cultivars under *in vitro* conditions should be investigated in more details in the future studies. Moreover, beside for resveratrol, investigation of stilbene compounds derived from resveratrol should be also considered. Moreover, *in vitro* conditions should be examined related to controlled breeding conditions and vineyard conditions to be better determination of genotype effect for resveratrol production. Expected resveratrol concentration in the latest commercial production for grape should be combined with “genotype x ecology” interaction which is getting more and more importance concept recently. Thus it is expected that studies about branding of both native genotypes and vineyard regions will be advanced.

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