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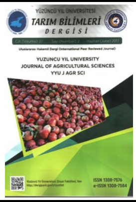

Araştırma Makalesi

Developing Functional and Antioxidant-Rich Table Grape Cultivars by Crossing Alicante Bouschet x Alphonse Lavallee

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Abstract: Recently, red and black colored grape cultivars are getting more popular due to anthocyanins in the skin and flesh of berries which are called teinturier grapes. Anthocyanins are a group of polyphenolics which can prevent many diseases by inhibiting or delaying the oxidative damage. But there is no a teinturier table grape cultivar in Turkey, but there are some teinturier wine grape cultivars such as Alicante Bouschet. Main goal of this study was to obtain tenturier genotypes by crossing of Alphonse Lavallee and Alicante Bouschet grape cultivars and to compare anthocyanin coloration of cotyledon and true leaves for early selection for tenturier candidates. Anthocyanin coloration was visually observed in cotyledon leaves. Amount of anthocyanin were determined in true leaves by spectrophotometrically disc method. Cotyledon and true leaves of most genotypes had the green color and there were parallelism between both leaves. The amount of anthocyanin of true leaves weredetermined as 50.21 mg/kg in green colored leaves and 131.51 mg kg^{-1} in dark colored leaves.

Alicante Bouschet x Alphonse Lavallee Melezlemesi Yoluyla Fonksiyonel ve Antioksidanca Zengin Sofralık Üzüm Çeşitlerinin Geliştirilmesi

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Öz: Son zamanlarda kırmızı ve siyah renkli üzüm çeşitleri, tanelerinin kabuk ve etindeki antosiyaninler nedeniyle ki bunlar tentüriye üzümler diye bilinir, gittikçe popüler olmaktadır. Antosiyaninler fenolik maddelerin bir grubu olup oksidasyon zararını engelleyerek veya geciktirerek birçok hastalığı önleyebilir. Türkiye'de Alicante Bouschet gibi bazı tentüriye şaraplık üzüm çeşitleri olmasına karşın tentüriye sofralık bir üzüm çeşidi yoktur. Bu çalışmanın ana amacı Alphonse Lavallee ve Alicante Bouschet üzüm çeşitlerinin melezlenmesi yoluyla tentüriye gnotipler elde etmek ve tentüriye çeşit adaylarının erken seleksiyonu için kotiledon ve gerçek yaprakların antosiyanin renklenmesini karşılaştırmaktır. Antosiyaninler kotiledon yapraklarında görsel olarak gözlenmiştir. Gerçek yapraklarda antosiyanin miktarı spektrofotomerik olarak disk yöntemiyle saptanmıştır. Çoğu genotipin kotiledon ve gerçek yaprakları yeşil renktedir ve her iki yaprak arasında paralellik vardır. Gerçek yapraklardaki antosiyanin miktarı yeşil renkli yapraklarda 50.21 mg/kg, koyu renkli yapraklarda ise 131.51 mg/kg olarak saptanmıştır.

1. Introduction

Grape is one of the highest economic importances of fruits with an output capacity of 75 million tons in the world (Anonymous, 2017). It is the most produced fruit crops with an amount of 4 million tonnes in Turkey. In this respect, grapes are the most popular fruit crops in the world and in our country, and they have great economic prospects. In addition to meeting domestic consumption in our country, it also provides an important foreign exchange contribution to the country's economy with approximately 400 million $ seedless raisins and 200 million $ table grape exports. There are around 15.000 grape varieties in the world and this number is increasing every year with the newly developed varieties of grapes. These varieties are divided into groups such as wine, table, dried fruit, and syrup. 71% of the world grape production is used as wine, 27% as table and 2% as dry (Guan, 2014). In our country, 25% of the production is consumed as a table grape (Arslan, 2016; Anonym, 2017). Grape is one of the top four fruits produced the most in the world. Turkey ranks fifth in vineyard area with 480.000 ha and sixth in grape production with 4 million tonnes in the world One-third of the annual production of grapes are exported as dried or fresh grapes. Turkey ranks first in exports of raisins, and sixth in terms of table grape exports in the world (Anonymous 2017; Seccia et al., 2015). The grape produced in Turkey is the second most fruit exported after hazelnut, as well as domestic consumption. Through this export, there is a foreign exchange inflow of about US$ 600 million in our country every year. From this point of view, viticulture is an agricultural branch with high economic importance, directly related to a wide range of producers in Turkey. There is a potential to increase these exports by developing new varieties for consumer demand.

The phenolic materials contained in the plants determine the antioxidant capacity of that plant. Antioxidants scavenge free radicals which are the result of metabolic events occurring in the body and are extremely harmful to human or plant health. Phenolic substances contained in fruits that determine antioxidant capacity. Grape is an extremely beneficial fruit because of the high and varied phenolic contents. Phenolic materials in grapes are mostly concentrated in skin and seed. However, 80-85% of the berry consists mainly of pulp (Çelik, 1998). For this reason, in addition to the skin and the seeds; it is possible to increase the antioxidant capacity of grapes by developing new varieties with phenol-rich in berry flesh.

Flavonoids are the main group of phenolic substances. One of the most important classes in this group is the anthocyanins. These substances give the plant parts their black or red color. They are mostly found in nature as glycoside forms of anthocyanidins. Anthocyanins are the largest group of flavonoids in grapes followed by flavonols such as quercetin, kaempferol, myricetin. The varieties with red colored fruit flesh are called the blood group in the horticultural crops as it is in the blood orange. In grapes, varieties with red flesh color are called teintürier grapes. This group of grapes is mostly found as wine grapes and it is used to enrich the red color in wines. The most famous wine grape cultivar of this group is Alicanthe Bouschet. Fruit flesh is usually colorless in table grapes. There is no teinturier table grape cultivar with economic importance in the present. However, due to the high antioxidant content of these grapes, new grape cultivars with tenturier characteristics are needed by consumers and producers. There is no such kind of grape in our country. Although there are teinturier wine grape cultivars available from the Vitis catalog databases, there is no data on tenturier table grape variety with economical value.

Foods that are more useful for health and high in antioxidant capacity are often referred to as functional foods. Similarly, grapes with rich phenolic substances and high in antioxidant capacity are called functional grapes. One of the ways to get antioxidants from grapes is to consume them as wine. However, it is not recommended to drink too much because of the negative effects on human health. The most healthy and safe way to get antioxidant substances from grapes is to consume them as a table grape.

Among the standard table grape cultivars, Alphonse Lavallee is widely grown due to its high yield and quality of grapes. Most of grape varieties have colorless (or white) berry flesh. However, there are some varieties such as Alicanthe Bouschet with a small number of red fruit flesh, and this group of grapes is called teinturier (Çelik et al., 1998). The Teinturier word is mostly used as a "tentüriye" in Turkish. These groups of grapes are all wine grape varieties and are used to improve the red color intensity of wines.

1.1. Tenturier grape breeding

The most important breeding method used in viticulture is hybridization. The maternal and paternal parents are crossed to combine targeted characteristics. Following hybridization, seeds are germinated and seedlings are grown to obtain hybrid plant populations. Then, each genotype is examined whether it carries the desired characteristics (Demir, 1975). Since each hybridization has its own distinctive genetic properties, their germination and seedling development characteristics are also different. The germination characteristics of grape seeds show a wide variation depending on the genetic structureof grape varieties.

Spiegel-Roy et al. (1980) obtained very large berries, up to 11.4 g, when the Alphonse Lavallee variety was used as the maternal parent in the hybrid table grape breeding programme. They emphasized that fruit juice is a transferable property to hybrids. In terms of skin color, Alphonse Lavallee is generally thought to be homozygous. Migicovsky et al. (2017) reported that development of a new grape variety by using traditional breeding methods took 25-30 years. However, early selection with markers linked to phenotypic features has shown that this process can be reduced by up to 10 years and that marker-assisted selection can reduce costs by 16-34%.

1.2. Antioxidants

About 8,000 phytochemicals were determined such as phenolics, alkaloids, carotenoids, nitrogenous compounds, organic sulfur compounds in plants. Phenolics are the largest and most common group in grapes and followed by carbohydrates and acids. Phenolics are divided into two main groups as flavonoids and non- flavonoids. One of the most common groups of flavonoids is anthocyanins (Yang and Xiao, 2013). Anthocyanins are an important quality factor that gives red and black color to fruit and is usually found in the skins of fruits. There are 17 anthocyanidins in the nature, 6 of which are more common. Main anthocyanidins found in high plants are cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin. These are usually called anthocyanins which are sugar-bonded to C_3 carbon atoms. Once the anthocyanins are synthesized in the cell, they are transported to the intercellular space. The skin color is determined by the concentration and type of anthocyanin. It is influenced by the cultural practices involved and environmental conditions although it is determined by the genetic structure of the variety. Ambient conditions such as light are very effective in the formation of anthocyanin. However, this situation is at different levels according to varieties. Low temperature, UV radiation, moderate drought, low nitrogen and phosphor content increase the formation of anthocyanins. In addition, from the hormones, ethylene and abscisic acid were positive; auxin and cytokinin are effective in the negative direction. There are differences in anthocyanins even in clones belonging to varieties. Anthocyanins in skins and fleshes may show differences. Malvidin derivatives in the skin and peonidin derivatives in the flesh are dominant. The concentrations of anthocyanins differ during the development period of berries. They increase rapidly after verasion. Although anthocyanins are usually found in skins in addition to berry flesh, cluster stalk, cluster skeleton, shoot, and leaf. The amounts of anthocyanins in young and very old leaves are higher than that of mature leaf. In the majority of the grape varieties, the fruit flesh is colorless and free of anthocyanins. Grape varieties containing anthocyanin in the skin and flesh of berries are called tenturier grapes like Alicanthe Bouschet variety.

There are more than 600 anthocyanins in nature, varying in their hydroxyl, aromatic and aliphatic acids; the number of sugar groups and the structure of the sugars according to their binding position. The amount and composition of the anthocyanin are affected by species, varieties, seasons, regions, maturity level, vegetative hormones, environmental conditions and cultural processes. Monoglycoside anthocyanins are found in *vinifera* grapes, diglycosid anthocyanins are found in American species. This feature is used in distinguishing wines. Diglycosides make wines more stable, while monoglycosides make it darker red. Anthocyanins are not stable and are subject to degradation which is affected by many factors such as pH, temperature, structure, light, oxygen, solution, enzyme and metal ions. The optimum temperature for anthocyanin biosynthesis is 25°C. In contrast, temperatures as high as 35 ° C accelerate the disintegration of anthocyanins and prevent their accumulation. It also prevents the genes involved. As a result, few anthocyanins are formed (He et al. 2010).

According to Canbaş and Kelebek (2008), Ribeerau-Gayon et al. indicated that only glucose is found in the structure of anthocyanin in grapes**,** and also they emphasized that anthocyanins are monoglycosides in vinifera and diglycosides in American species.

1.3. Inheritance of anthocyanins

It is stated by Guan (2014) that the anthocyanins inheritance is controlled by a single gene pair in grapes. In addition, skin and flesh colors are controlled by a major gene and many minor genes. Major gene determines the color of skin and flesh, while minor genes determine the concentration of anthocyanin in the skin. When paternal teinturier cultivar was crossed with maternal cultivar which has colorless fruit flesh, all skin colors were red or black but flesh color segregated as 1:1(50% tenturier+50% colorless) in F_1 hybrids. It is indicatedthat fruit flesh is at heterozygote structure and colorlessness is a recessive feature in grapes. Liang et al. (2011) presented that total amount of anthocyanin increases with the level of ploidy, so the genetic background (parents) is very important in the accumulation of anthocyanin compounds in the offspring. The researchers stated that the color was controlled by two pairs of genes in grapes and introduced firstly by Barrit and Einset in 1969. If the B and R genes are dominant, it is claimed that black and red grapes are formed respectively and if both genes are recessive white grapes are formed. However, Spiegel Roy et al. (1980) claimed that the grape color was dominated by a single gene pair and the white color was recessive.

Liang et al. (2009) identified 16 anthocyanins, all of which are monoglycosides, in a study of three different vinifera populations. The maternal parent expressed that the proportions of existing anthocyanins determine the proportions of anthocyanins of the hybrid progeny but do not determine the amount. Anthocyanins in the grape skin that heredity is governed by many genes and it is a quantitative character with high heritability level. According to the same researchers, Deng and Qu found that malvidin and petunidin derivatives are purple; pyridin and cyanidin derivatives are red and the delphinidin derivatives are blue in color. The density of these colors is dependent on the concentration of anthocyanin derivatives in the skin. Likewise, Shi et al. emphasized that the black and purplish red color are dominant; red and purple color heterozygote whereas white color is homozygous recessive. Furthermore, they explained thatthe main factor in determining the amount of anthocyanin is genetic but it is affected by climate conditions and may vary with years. However, derivatives of delphinidin and petunidin changed less are remained constant throughout years. Temperature, sunlight, low humidity, and rainfall from the climate factors have a positive effect on the synthesis of all anthocyanins. As a result, researchers have determined that the presence or absence of anthocyanins in the grape skin is controlled by major genes in the maternal parent, but that the amount of these anthocyanins is controlled by minor genes. For this reason, the breeders emphasized that when selecting a parent, they should pay attention to the characteristics of the anthocyanin of the variety. For example, table grape consumers have chosen bright and vivid grapes, and therefore parents with cyanidin and peonidin derivatives should be selected for table grape breeding programmes, while varieties containing malvidin derivatives should be used in wine grape breeding studies in order to give attractive bright red and purple color in wine. They emphasized that paternal parents determined the level of the anthocyanin in the hybrid.

Grapes vary from slightly pink to dark brown depending on the amount of anthocyanin deposited in skins or white in color. The difference in the amount of anthocyanin in the grapes was revealed by the three VvMybA genes in a single gene cluster. Rapidly developing molecular studies on grape breeding in the last few decades have revealed that black or red color is controlled by the AMBNY gene in the grapes and that it is dominant. The white color of the grape varieties is caused by mutations that occur in the VvmybA1 gene which controls the formation of anthocyanins. All white grapes have a common origin (Walker et al., 2007; Fournier-Level et al., 2009; Xie et al., 2015).

Xie et al. (2015) stated that the red color of Alicanthe Bouschet began to appear in the flesh before the skin. They also reported that tenturier wine grape cultivar named as Yan 73 contains anthocyanin in the cluster stem and skeleton in addition to skin and berry flesh. They showed that there were more than 10 times of gene expression in the skin compared to the berry flesh. For this reason, they have explained that the amount of anthocyanin in the skin is higher than that of flesh.

In some types of teinturier grapes such as thick-skinned Violeta, the skin ratio was found to be as high as 46% of the whole grape berry. The amount of anthocyanin in Violetta was found to be 3950 mg/kg, and the vast majority of it came from the skin. On the other hand, Mazza also emphasized that the amount of anthocyanin in teinturier varieties increased up to 6030 mg/kg. However, it was lower in varieties which had black skin and white berry flesh (Rebello et al., 2013).

Main goal of this study was to obtain tenturier genotypes by crossing of Alphonse Lavallee and Alicanthe Bouschet grape cultivars and to compare anthocyanin coloration of cotyledon and true leaves for early selection of tenturier genotypes.

2. Materials and Methods

The trials were carried out between 2017 and 2018 at Akdeniz University, Faculty of Agriculture. Alicanthe Bouschet and Aphonse Lavallee grape varieties were crossed in early May 2017. Alicanthe Bouscet cultivars were used as maternal parent.The clusters were harvested on September 10, 2017 and seeds were extracted from berries. These seeds were stored in 5°C for 4 months in moist perlite. Then seeds planted in compressed peat pots (Ø6cm) were germinated in plastic boxes placed in 25°C room and transferred to the greenhouse when 5-6 true leaves were formed in plants. Observation of anthocyanin coloration in cotyledon leaves was followed by germination. Measurements of anthocyanin were carried out in hybrid plants with 10-15 true leaves. Mature true leaves of 4-6 nodium from shoot tip were taken for anthocyanin analysis.

2.1. Observation of anthocyanin density in cotyledon leaves

Anthocyanin coloration was observed by modified Becker (1986) method. In order not to interrupt the development of hybrid seedlings, cotyledon leaves were not removed from seedlings and coloration status was determined by visual observations. The evaluation of cotyledon coloration was made from the time of germination to the first true leaf emerged in the seedling. At this stage, the anthocyanin density in the cotyledon leaves of each genotype was evaluated between 1 to 5 scale values (1: none; 5: very dark). The seeds which hypocotyl hook was observed at the surface of soil were considered to be germinated. The germinated plants were grown in plastic boxes under artificial light (10 Wm-2), at a temperature of 26°C and 95% relative humidity.

2.2. Anthocyanin measurements in true leaves

This method, also called leaf disc test (Pirie and Mullins ,1976; Carbonneau,1980; Ramming and Cousins,2014). Following the germination of the hybrid seeds in the germination chamber and the formation of true leaves in the growth chamber, plantlets were transferred into the greenhouse. The true leaves that were removed from the plant were surface sterilized with sodium hypochlorite (containing 6% Cl_2) and then washed with sterile distilled water. 9 pieces of 10 mm diameter leaf samples were taken from the leaves of each genotype in a sterile cabinet and placed in a sterile petri dish with a test solution containing 10% sucrose. The covers of the petri dishes were sealed with parafilm. Thus, a total of 27 leaf samples discs were taken from each of the genotypes, with 3 replications and 9 replicates per repetition. Petri dishes were placed in plant development room (Temperature: 27°C; photoperiod: 16 hours light+8 hours dark; illumination: 10 Wm^2) according to randomized plot design. The total amount of anthocyanin was determined 5 days after collection of leaf samples.

Total anthocyanins in true leaf samples were determined according to modified Pirie and Mullins (1976), and Cemeroğlu (2018) methods. The total amount of monomeric anthocyanin with the coefficient of the malvidine was calculated as mg/kg (Cemeroğlu, 2018). On the morning of the 6th day, the discs were washed with distillate water. All the discs in each repetition were weighed with precision scales and placed in 50ml volume falcon tubes. 15 ml of HCl (1.5N) and 85 ml ethanol (96%) were mixtured as extraction solution. The final volume of each tube was set to 10ml with extraction solution and macerated at room temperature by using Ultra-Turrax macerator (TP 18N head) operated at full speed. Homogenized leaf samples were kept in dark for 15 hours at 4°C and then centrifuged at 5 000 x g for 10 minutes. Supernatant of 0.5 ml were pipetted into each of 2 glass tubes. First tube contains 0.5 ml supernatant and 7.5 ml potassium chloride buffer solution (pH 1). Second tube contains 0.5 ml supernatant+ 7.5 ml sodium acetate buffer solution (pH 4.5). Each tube homogenized with vortex for 1 minute. The values of malvidin-3 glycoside absorbance 520 nm and 700 nm values were recorded in both buffers in the spetrophotometer. Results were expressed as mg/kg malvidin in true leaves.

3. Results

As a result of the hybridization study, a total of 284 hybrid plant genotypes were obtained. The data on the coloration of cotyledons and true leaves of genotypes are given in Table 1. In the majority of genotypes (47.5%), it was observed that the leaves of cotyledon did not contain anthocyanins, ie they were green in color and were in scale 1. As the scale value increased, so the anthocyanin coloration in the cotyledon leaves became darker, the percentage of the genotype entered into each group decreased gradually from 47% to 5%. The anthocyanin amount of true leaves were determined when the plants had shoot length of 30-40 cm and all leaves had greeen color. However, these leaves may form anthocyanin in the sucrose solution. Then they were compared with the anthocyanin coloration of cotyledon leaves of same genotype (Table 1).

Table 1. Anthocyanin coloration of cotyledon and true leaves of hybrid genotypes

Characteristics	Scale of anthocyanin coloration in cotyledon leaves					Total
	1	2	3	4	5	
Number of plant (piece)	135	74	40	28	7	284
Percentage of genotypes in each scale group (%)	47.5	26.1	14.1	9.8	2.5	100
Amount of anthocyanin in true leaves (mg/kg)	50.21 b	90.83 ab	86.28 ab	100.84 a	131.51 a*	-

*Means within line followed by different letters differ significantly by Tukey test ($P < 0.01$).

According to the disc method, genotypes with cotyledon leaves in green color (Scale no:1) were found to have a small amount of anthocyanin in the sucrose medium (50 mg/kg). Although the anthocyanin content of the anthocyanin coloration in cotyledon leaves varies from 1 to 3, the amount of anthocyanin in the true leaves of the genotypes was different from the absolute value, but there were no significant differences statistically. Whereas, statistically significant differences were found between genotypes with cotyledon leaves green and anthocyanin amounts formed in the true leaves of dark and very dark genotypes. The amount of anthocyanin was 50,21 mg/kg in green colored true leaves and 131.51mg/kg in dark-colored true leaves.

3. Discussion and Conclusion

It is stated that grapes of hybrid genotypes can give an idea about whether varieties of berry flesh coloration will be present in the future and will allow early selection in hybrid plants (Pirie and Mullins, 1976; Carbonneau, 1980; Ramming and Cousins, 2014). This hypothesis has been tested in the germination and seedling stage of Alicanthe Bouschet x Alphonse Lavallee crossing whether it will work in hybrid plants but it is not yet completed. When the same genotypes form grape clusters, the berries should be compared with the fruit flesh coloration. At this stage, cotyledon leaves and true leaves show parallelism. Pirier and Mullins (1976) indicated that Absicic acid and sucrose increase anthocyanin accumulation in grape leaves.

Briefly, the relationship anthocyanin coloration between cotyledon and true leaves of hybrid genotypes was demonstrated in this tenturier grape breeding study and the following conclusions were reached:
1. The green true leaves may contain a small amount of anthocyanin inside.
2. There is a parallelism between cotyledon leaves and anthocyanin coloration of true leaves.

3. The majority of cotyledon and true leaves of hybrid genotypes were green or have very little anthocyanin coloration was detected in Alicanthe Bouschet x Alphonse Lavallee crossing.

The study should be continued after the plants form the cluster. There is a need to reveal the relationship between the coloration status of the grapes of hybrid genotypes and the anthocyanin coloration in cotyledon leaves and their true leaves in the seedling stage.

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