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Research Article

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A New Method to Determine the Stomata Density from Transparented Vine Leaves

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Abstract In this research, it was aimed to determine stoma density of Semillion, Gamay, Narince, Michele Palieri and Çavuş cultivars and 3309 C, SO4, 5BB, 41B and 1103P rootstocks leaves which was grown in Tekirdağ Viticulture Research Station. Two methods were used; transparented leaf method and nail polish method. These two methods were compared in point of stomatal density. Stomatal density changed between 276.04 ± 5.31 and 170.58 ± 4.03 stoma/mm^2 at cultivars. At rootstocks, stomatal density changed between 256.25 ± 15.83 and 172.27 ± 3.55 stoma/mm^2. No significant differences were found between transparented leaf method and nail polish method in terms of counting the number of stomata. But when we compare two methods, it was concluded that the number of stomata which detected in the leaf area is higher at transparented leaf method than nail polish method.

Consequently, it was found that determining stomatal density with using transparented leaf method was more reliable than nail polish method.

Keywords Grapevine, leaf, nail polish method, stoma density, transparented leaf method

Introduction

Stomas are small pores that provide the input of CO_2, O_2 and water vapor into plants and are densely found in the above-ground parts of plants, especially in leaf epidermis [1].

On the lower surfaces of the vine leaves, there are many stomata that allow the regulation of the gas exchange necessary for the photosynthesis and the evaporation of the water. The vine, which is located between the soil and the atmosphere, continues its life activities through stomata in its leaves. 80-85% of the total transpiration in plants is done with the help of stomas. In addition, the stomas, which are capable of opening and closing regularly, are opened when necessary, allowing the necessary gas exchange for photosynthesis, and prevents unwanted water loss. These characteristics of the stomas reveal that they have very important effects on the physiology, adaptation and efficiency of the plants and thus they become an indispensable part of the life chains of the plants [2].

Unlike normal epidermis cells, the stomas contain chlorophyll and composed of two cells in the form of kidney. These structures are usually found on the lower surface of the leaves (hypostatic leaf), but sometimes they can only be located on the upper surface of the leaf (epistomatic leaf). In some cases, stomas can be found both the upper and lower surface of the leaf (amphistomatic leaf) [3].

The size and density of the stomata vary according to plant species and varieties and plant growing conditions. In a study conducted on the leaves of fourteen Vitis species and varieties, it was determined that there were differences in the stoma density between species and varieties, but there was no significant difference between stoma numbers in different lobes of the leaf [4].

It is determined that grape varieties which grafted on different rootstocks differ from each other in terms of stoma densities [5].

Resistance to dryness in rootstocks and cultivars is closely related to the number of stomata in the leaf. The number of stomata in the leaves of drought-resistant rootstocks is usually small. The number of stomata in the leaves of the culture varieties grafted on such rootstocks shows a decrease in the number of stomata and thus the resistance to drought increases [6].

As a result of studies on varieties of apples and figs, it has been revealed that there are differences in the number of stomata among the varieties [7-8].

Stoma densities of Red Globe, Razaki, Flame seedless and peace types were determined on the lands of Eğridir Horticultural Research Institute. The number of stomata between varieties ranged from 109.8 to 153.8 in mm^2. While the lowest number of stomata was taken from the Peace type with 109.8 stomata/mm^2, the highest stoma value was obtained from Red Globe variety with 153.8 stomata / mm2. Stoma densities varied according to variety [9].

There are studies on the existence of the relationship between the resistance of plants to various environmental conditions and stomata. At Sultani Çekirdeksiz grape variety, in the absence of anhydrous conditions, stomata have been found to rapidly block transpiration and photosynthesis to adapting plants to drought. As a matter of fact, it is reported that water deficiency in soil causes an increase in the number of stomata and it is an indicator of xeromorphic feature [10]. Similarly, it is noted that the cold resistant varieties have fewer stomata than the sensitive varieties. These ecophysiological features show that stomata play a very important role in the investigation of the factors such as viticulture, especially drought resistance, and in the classification of the varieties in this subject and in the acquisition of new varieties.

The successful cultivation of horticultural crops depends on plant-water relations, like other conditions. The stomata in the leaves play a big role in the regulation of these relations. Close to 85-90% of the water loss in plants is occurs by stomas. Therefore, the number and structure of stomata in the leaves of each culture plant should be known [11].

In this study, the number of stomata taken from the leaves of different cultivars and rootstocks were compared. Also, in this study, counting has been done with the method of transparented leaf and the nail polish method which are the methods of stoma counting, and the relationship between these methods has been examined.

Materials and Methods

5 grape varieties (Semillion, Gamay, Narince, MichelePalieri and Çavuş) and 5 rootstocks (3309 C, 5BB, SO4, 1103P and 41B) were selected as materials to compare and detect differences of the stoma density in the unit area (mm^2). The samples were taken from the shoots of the selected cultivars and rootstocks, from the leaves of the normal form, which were not solidified, disease-free, and cultivated in the 4th to the 5th knots. 5 samples were taken (from the 4th and 5th knots) from each variety and rootstock.

Counting of stomata by nail polish method

Transparent nail polish was applied with a special brush to the area of 1.0 × 3.0 cm to the right and left of each main vessel from bottom to top of the main veins of the leaf. After the nail polish was dried, the stoma numbers in the quadratic ocular micrometer were determined with microscope × 20 magnifications by removing the pattern on the leaf with selloteyp [2].

Counting of stomata by transparented leaf method

Samples were taken from each of the leaf samples with a special circular punch with a diameter of 1.50 cm from the left and right sides of the veins along the main veins on the underside of the leaf. 10 pieces of circular pieces were taken from each leaf (Figure 3.) and placed in the solution.

Transparency solution

Sodium hypochlorite (2.5%) solution was used to make transparent leaf samples. Circular pieces from the leaves were placed in solution. The samples kept at room temperature (22-23 °C) for 10-12 hours were completely

transparent (Figure 4) and then taken onto the slide. The samples were counted in × 20 magnification microscope and the obtained values were multiplied by 0.24 coefficient and the stoma count in mm2 was obtained from the proportional calculations.

Sodium hypochlorite

Sodium hypochlorite (NaClO) is a type of salt. It is used in bleaching in daily life. Also a certain amount of sodium hypochlorite, makes the leaf transparent when applied (Figure 5). This substance is produced by combining sodium hydroxide and chlorine in room conditions. Formula for obtaining a sodium hypochlorite is as follows:

$$2NaOH + Cl_2 \rightarrow NaCl + NaClO + H_2O$$

Measurement, counting, analysis and evaluation

5 samples were randomly selected for each variety from the leaves which were molded and transparent. The leaf samples were analyzed in four different areas of the leaf samples with 4 iterations. Variance analysis was performed according to the randomized block design with 4 replicates. As a result of the variance analysis, the difference in the stoma density between the same varieties and in varieties was investigated at LSD (0.5%) level. Between the two methods, the differences in the number of stomata were investigated. Stoma numbers are shown in the charts as stomata/mm^2. The obtained results are explained on the charts and their values are shown as histograms and photographs taken under the microscope are included.

Results

Comparison of two methods in the leaves of cultivars

As a result of the measurements made by both methods; in terms of the number of stomata in the unit area, differences were observed. Çavuşvariety had the lowest stoma density with 170.58 ± 4.03 stomata/mm^2, while the M. Palieri variety had the highest stoma number with 276.04 ± 5.31 stomata/mm^2. Between these two values were; Semillion 205.21 ± 6.31 and 192.46 ± 11.46 stomata/mm^2, Gamay 213.04 ± 9.63 and 208.58 ± 8.62 stomata/mm^2, and Narince 251.04 ± 13.90 and 242.96 ± 4.61 stomata/mm^2 (Table 1).

Table 1: Comparison of stoma density detected in leaves of cultivars with transparented leaf and nail polish methods

Cultivars	**In leaves of cultivars**						
	Transperented leaf method (Stomata/mm^2)	**(*)**	**(**)**	**Nail polish method** (Stomata/mm^2)	**(*)**	**(**)**	**Method difference** (Stomata/mm^2)
Semillion	205.21 ± 6.31	c	a	192.46 ± 11.46	b	b	2.75
Gamay	213.04 ± 9.63	c	a	208.58 ± 8.62	c	a	4.46
Narince	251.04 ± 13.90	d	a	242.96 ± 4.61	d	b	8.08
M. Palieri	276.04 ± 5.31	e	a	270.58 ± 5.54	e	a	5.84
Çavuş	172.42 ± 5.68	a	a	170.58 ± 4.03	a	a	1.84
(*): Between cultivars (**): In cultivars (LSD: %5)							

The difference in the number of stomata was found to be statistically insignificant between the measurements made by the method of transparented leaf and nail polish method in the vine leaves of the varieties. However, measurements by using the method of transparency were found to be higher than the nail polish method by number of stomata. For example; in M. Palieri variety, the number of stomata was found in the transparented leaf method was 276.04 ± 5.31 stoma/mm^2 and in the nail polish method was 270.58 ± 5.54 stoma/mm^2.

When the comparison was made between the varieties, the difference in the number of stomata between the varieties was found statistically significant except Semillon and Gamay (Figure 1).

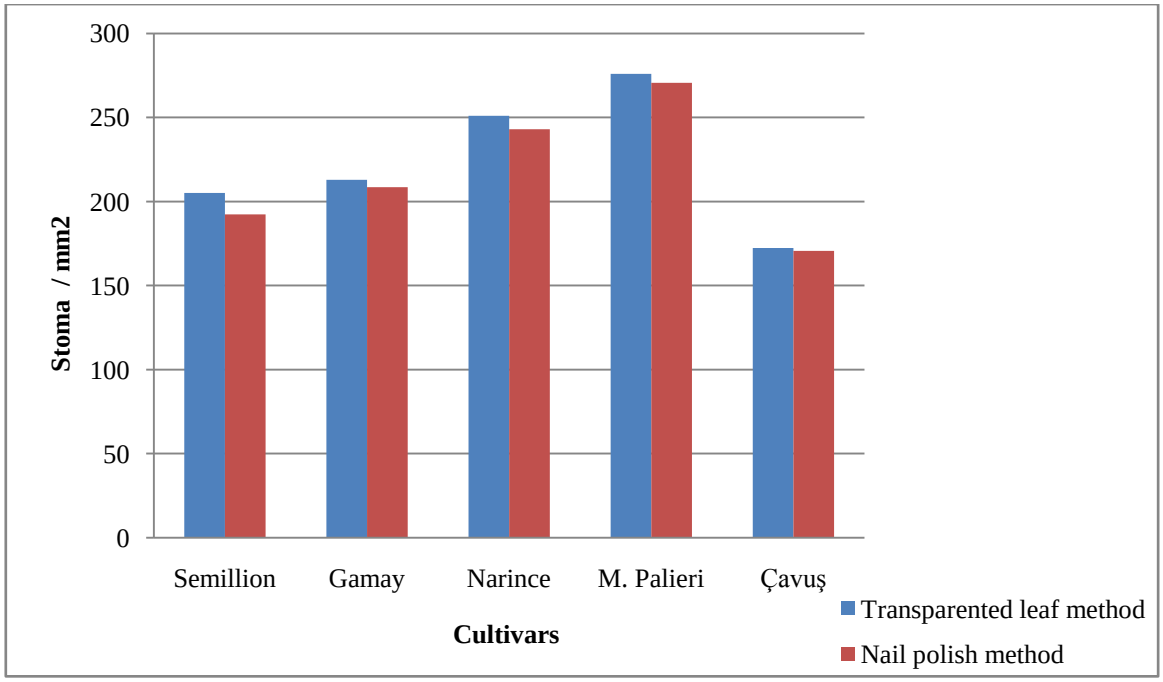


Figure 1: Comparison of number of stomata between transparent leaf method and nail polis method in leaves of culture vine varieties

Comparison of two methods in the leaves of rootstocks

As a result of the measurements made by both methods, there were a difference number of stomata in the unit area between rootstocks. The 1103P rootstock had the lowest stoma density with 172.27 ± 3.55 stomata/mm^2 while the SO_4 rootstock had the highest stoma number with 256.25 ± 15.83 stomata/mm^2. Between these two values were; 41B187.25 ± 3.20 stomata/mm^2, 5BB 228.91 ± 4.91 and 223.46 ± 9.54 stomata/mm^2, and 3309 C, 255.78 ± 6.53 and 246.38 ± 6.28 stomata/mm^2 (Table 2).

Table 2: Comparison of stoma density detected in leaves of rootstocks with transparented leaf and nail polish methods

Root stocks	**In leaves of rootstocks**						
	Transperented leaf method (Stomata/mm^2)	**(*)**	**(**)**	**Nail polish method** (Stomata/mm^2)	**(*)**	**(**)**	**Method difference** (Stomata/mm^2)
3309 C	255.78 ± 6.53	D	a	246.38 ± 6.28	d	b	9.40
5BB	228.91 ± 4.91	c	a	223.46 ± 9.54	c	b	5.45
SO4	256.25 ± 15.83	d	a	247.92 ± 10.87	d	b	9.33
41B	187.25 ± 3.20	b	a	-	-	-	-
1103P	174.75 ± 3.71	a	a	172.27 ± 3.55	a	b	2.48

(*): Between rootstocks (**): In rootstocks (LSD: %5)

The difference in the number of stomata was found to be statistically insignificant between the measurements by using the methods of transparented leaf and nail polish. However, measurements using the method of transparented leaf were found to be higher than the number of stomata measurements. For example; while the number of stomata in the SO_4 rootstock intransparented leaf method was 256.25 ± 15.83 stomata/mm^2 and in the nail polish method was 247.92 ± 10.87 stoma/mm^2.

When we compared the number of rootstocks with each other in terms of number of stomata, the difference in the number of stomata between rootstocks was statistically insignificant in the rootstocks of SO_4 and 3309 C, while it was found to be significant in other rootstocks (41B, 5BB, and 3309 C). Same results were found in transparented leaf method and nail polish method (Figure 2).

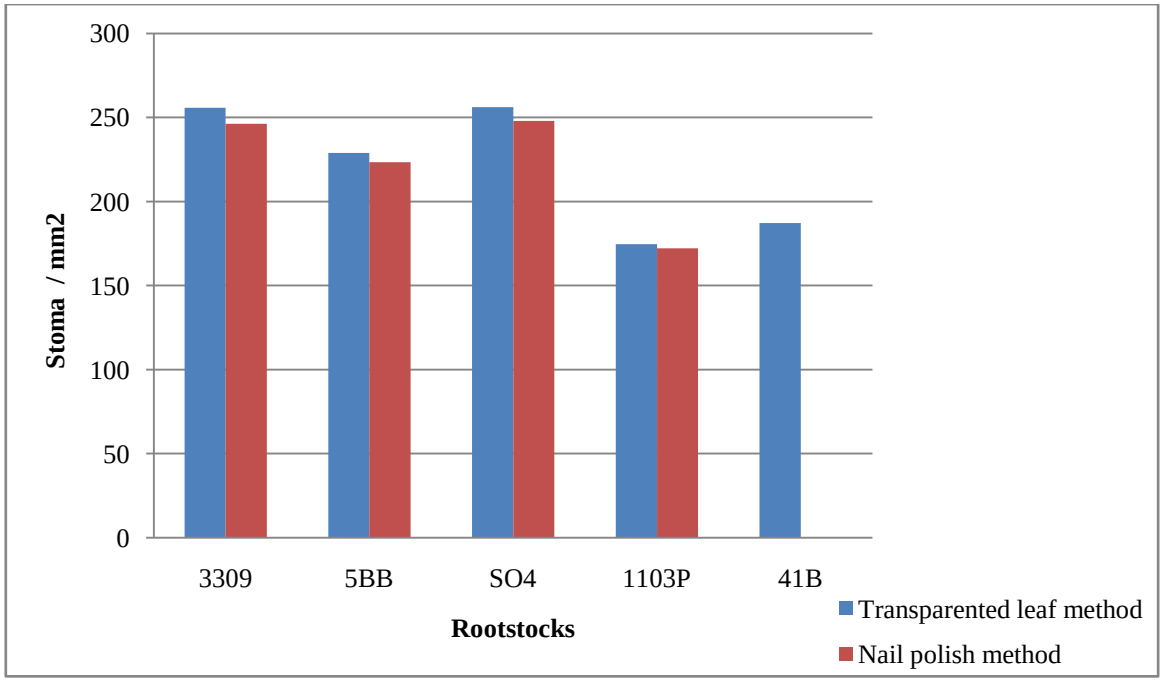


Figure 2: Comparison of number of stomata between transparent leaf method and nail polis method in leaves of rootstocks

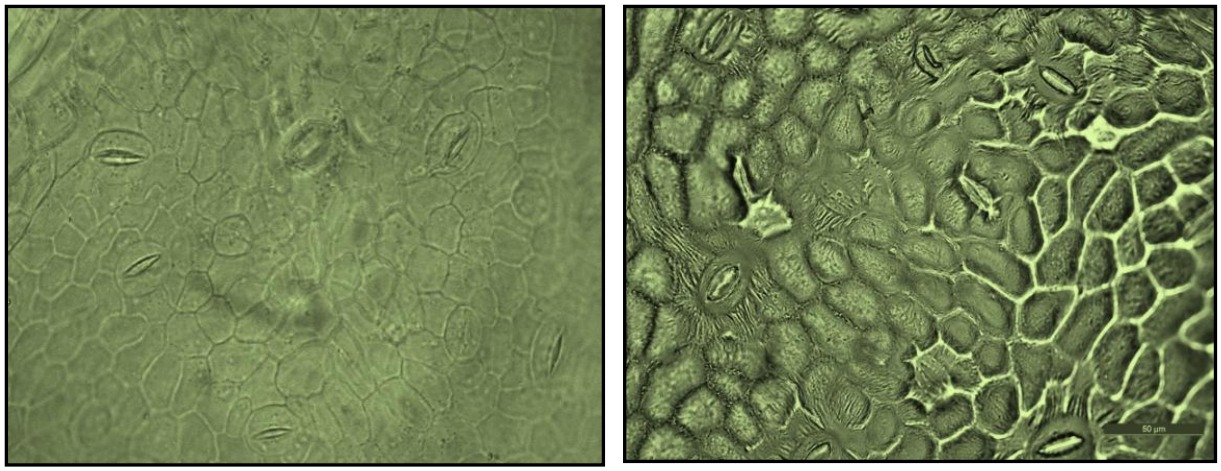
Images from Microphotographs and Comparison

It is also observed in the pictures that the number of stomata of the leaves changes between the varieties and rootstocks, where the number of stomata is a characteristic to vary. It is also seen in the microphotographs that the samples taken using the transparented leaf method contain more stoma than the samples obtained by nail polish method (Figure 6, Figure 7, Figure 8 and Figure 9).

Figure 3: Taking samples with a special circular punch 1.50 cm in diameter from the left and right sides of the veins along the main veins of the leaf

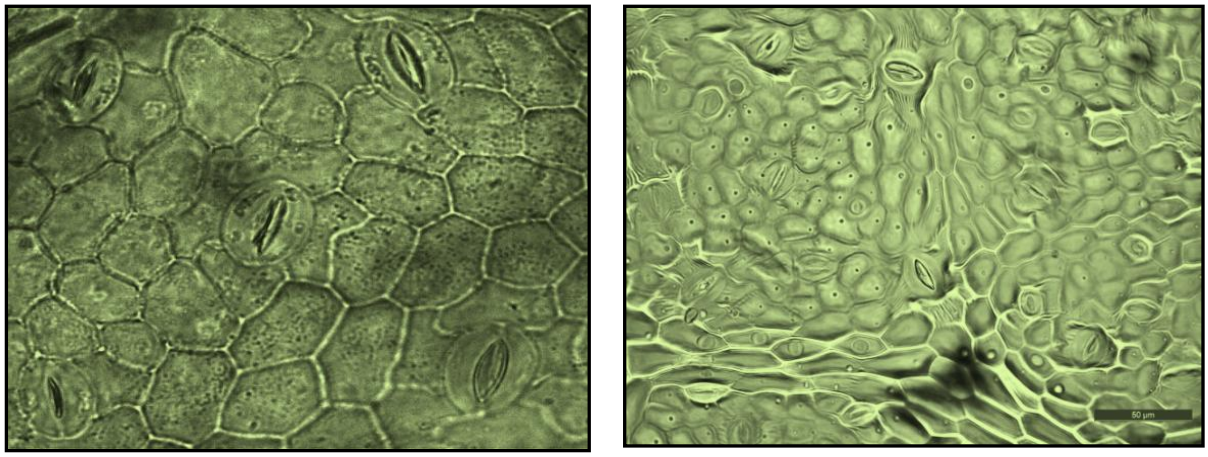
Figure 4: Placing circular green leaf samples in Sodium hypochlorite (2.5%) solution (left). After circular leaf samples kept at room temperature (22-23 °C) for 10-12 hours, are completely transparent (right)

Figure 5: A complete transparent leaf of vine which sodium hypochlorite (2.5%) applied on and kept at room temperature (22-23 °C) for 10-12 hours



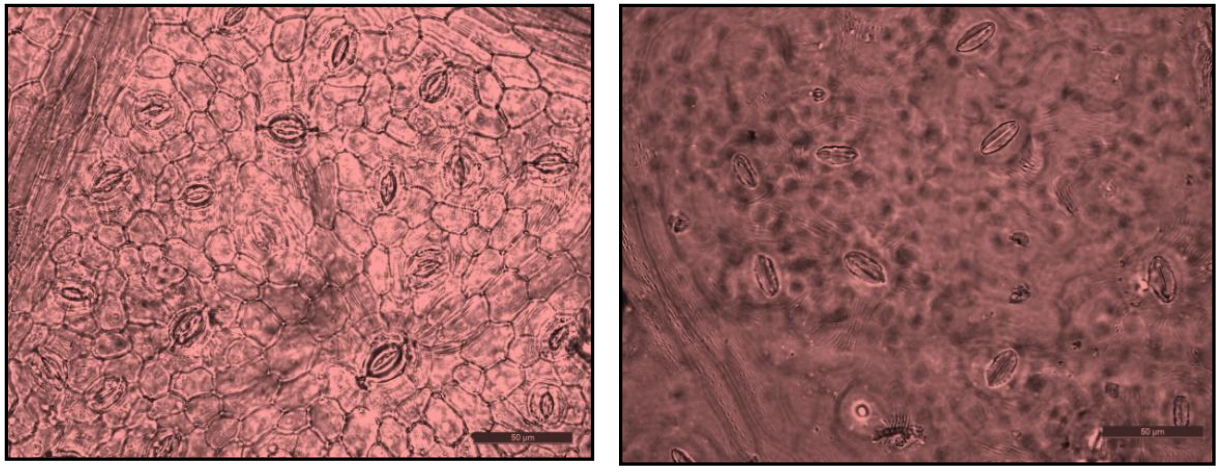
(a) *(b)*

Figure 6: The appearance of the leaf of Semilion under the microscope (10 × 40) with the method of transparented leaf (a) and nail polish method (b).



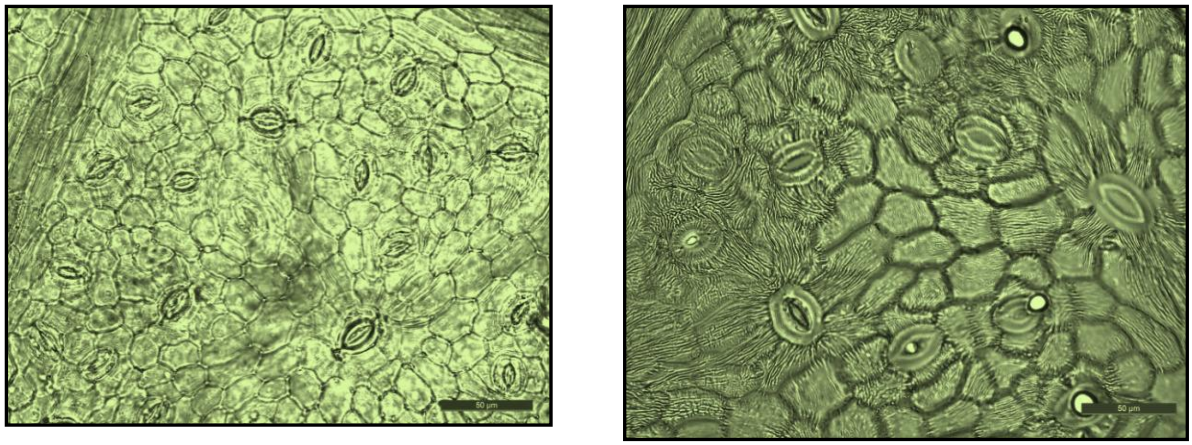
(a) *(b)*

Figure 7: The appearance of the leaf of Gamay under the microscope (10 × 40) with the method of transparented leaf (a) and nail polish method (b).



(a) *(b)*

Figure 8: The appearance of the leaf of SO_4 under the microscope (10 × 40) with the method of transparented leaf (a) and nail polish method (b)



(a) *(b)*

Figure 9: The appearance of the leaf of 3309 C under the microscope (10 × 40) with the method of transparented leaf (a) and nail polish method (b)

Discussion

The pores are located on the underside of the vine leaf and the density in the unit area (mm^2) varies according to the type and the cultural process applied [4].

Stoma density in the leaves changes by species, leaf sun and shade, the temperature of the air, altitude, air humidity and soil water.

The density of the number of stomas is a feature specific to the variety; It is stated that it is not possible to mention a definite difference in the number of stomata in irrigated and non-irrigated vines of the same type unless there are conditions under severe water stress [12].

The density of the stoma measured in the grape varieties is usually between 129-254 stoma/mm^2 and is reported by Eris and Soylu [2].

The stomas, which make up the most specialized cells of the epidermal tissue, are usually found on the lower surface of the leaf. Because the upper epidermal surface of the leaf is directly exposed to the sun, the temperature of the upper surface of the leaf is considerably higher than the lower surface. The high leaf temperature causes an increase in the transpirationrate; the stoma is located on the lower surface and away from direct sunlight reduces water loss. Wax accumulation and excess hairy leaf surface also reduce the effect of sunlight and transpiration rate [13].

It was found that there was no statistically significant difference between the methods in terms of the number of stomata. Since some of the stomas are found embedded within the fungal layer of mesophilic tissue through the leaf epidermis, these stomas are unlikely to leave traces in the nail polish method. Therefore, such a mold does not give accurate results in terms of the number of stomata. The nail polish used in the molding process is a chemical compound which contains formaldehyde acetate, butyl stearate, trimethyl pentanyl as the solvent, primarily nitrocellulose, resin and colorants from film-forming materials. Since this substance causes browning and deformation of the leaf when applied to the leaf, a complete mold containing stomas cannot be removed.

This method may be misleading in determining the actual number of stomata in the unit area as the stoma in the leaf mesophilic tissue on the lower surface of the leaf cannot be molded. In addition, because of the substances contained in the nail polish green colour of the leaf is being brown and stoma cannot be count from the mold. To determine the actual number of stomata in the unit area due to these drawbacks, the method of transparented leaf developed by Çelik and Nikolaou [14] was used and both methods were compared.

As a result of the measurements made by both methods, the difference in the number of stomata in the Çavuş variety was not statistically significant. However, it was determined that the number of stomata obtained in the measurements using the transparency method was higher than the method of nail polish. For example, the number of stomata was found 172.42 ± 5.68 stomata/mm^2 at transparency method, while the number of stomata was found as 170.48 ± 4.03 stomata/mm^2 at nail polish Method in Çavuş culture. Stomata are located on the lower surface (abaxial) of the vine leaf. They are covered with vertical and horizontal hairs. It may not be

possible to see all the stomas in the molds taken with nail polish in the varieties which form dense hairiness especially on the surface of the lower leaf like Çavuş. This makes it a suspect to reach the right conclusion.

As a result, it was concluded that the number of stomata is a characteristic to the variety, and it is not possible to speak of a difference in the number of stomata between the leaves of the same variety or rootstock under the vineyard conditions. Based on the findings, it was found that the number of stomata in the unit area counted by microscope did not reflect the exact number of stomata taken from the lower surface of the leaf using nail polish method. It has been concluded that the method of transparented leaf can be an alternative to the method of nail polish, which is a classical stoma counting method, and may replace the nail polish method for a more reliable and healthy study.

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