

MORPHOLOGICAL, ANATOMICAL AND PHYSIOLOGICAL LEAF TRAITS OF PISTACHIO (*PISTACIA VERA* L.) GROWN IN BUCHAREST AREA (ROMANIA)

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Abstract

Pistachio (Pistacia vera L.) is a very important crop species due to its nutrient-rich nuts, as well as its special ability to adapt to climate changes. Micromorphological, anatomical and physiological characteristics have been performed in the leaf taken from the male tree, grown in the Bucharest area (Romania). The samples, represented by mature leaves, were collected in June-July. Leaves are simple, but also trifoliate, with ovate leaflets, with entire margins, glossy. The leaf micromorphology was performed by scanning electron microscopy. For anatomical observations, epidermis was collected from both sides of the leaf, transverse sections were carried out in the leaf lamina and petiole, then observations have been done using light microscopy. Stomata are of anomocytic type, located on both sides of the leaf. The secretory ducts have been identified in the phloem of the vascular bundle of the petiole and in the middle vein of the leaf lamina. The leaf mesophyll is equifacial, having the palisade tissue under both epidermises, and the spongy tissue is situated in the middle area. Physiological indicators can be useful for the characterization of the pistachio adaptation mechanisms in the resilience integrated framework.

Key words: *Pistacia vera*, anatomy, morphology, physiology.

INTRODUCTION

The genus *Pistacia* is characterized by a special ecological and phenotypic plasticity (El Zerey-Belaskri, 2019), as well as considerable genetic diversity (Hakimnejad et al., 2019). Although it shows increased tolerance to the stress factors action, arid and semi-arid regions where it usually grows are known for exhibiting extreme stress conditions, especially regarding drought and salinity levels (Behboudian et al., 1986; Mehdi et al., 2011; Ben Hamed et al., 2023), which are higher than usual (Esmaeilpour et al., 2016b). Low temperatures can also be added, which in certain areas may impair the species performance (Ravari et al., 2023), as is the case in Romania. Thanks to the ecological plasticity, both at the individual and/or the population level, it is possible to adapt to the temporal and/or spatial variations of the different environmental limiting factors by developing some regulatory mechanisms which determine morphological and physiological changes, as recently demonstrated in *Pistacia lenticus* (Doghbage et al., 2023). The attention given to

it is also supported by a recent exhaustive review carried out by Nezami and Gallego (2023), which deserves to be studied carefully to create an ensemble image of this genus, from its origins to the present, and more than that on the perspective concerns on this topic.

Pistachio (*Pistacia vera* L.) is the most economically important species in the genus *Pistacia* (FAOSTAT, 2023; cited by Miri et al., 2023), recognized as such due to its unique nutrient composition, as well as its special ability to adapt to climate changes (Bailey et al., 2020).

Consequently, the knowledge of behavior in different conditions, the possibilities of adaptation to new environmental areas, as well as the increase of resilience to environmental challenges must be in the attention of researchers and farmers in the areas where this species is usually cultivated and why not also where its growth would be possible. As mentioned by Hakimnejad et al. (2019) after studying 42 genotypes belonging to different pistachio species (including *P. vera*), significant positive correlations registered between morphological, eco-physiological and photosynthetic indicators

can represent a starting point for improvement of the breeding programs. Notably, the 'Siirt' cultivar belonging to *P. vera* (included in the first group by cluster analysis) can be used in breeding programs as parents, to increase the degree of tolerance of rootstocks and cultivars to environmental conditions.

P. vera being a dioecious species and as it is well known, for such species one of the adaptation strategies related to sex (males and females) is represented by the structural and functional characteristics of the leaves (Korgiopolou et al., 2019). The research team conducted a comparative study on 50-year-old trees (females with low fruit load and males), both in sun and shade conditions. For the male trees, the leaves were smaller and total conducting petiole area (TCA) was significantly smaller, while the stomatal density, the water use efficiency and the content of phenolic substances had higher values. In the case of the female trees, the leaves had a larger surface, with a greater thickness, higher values of the leaf mass/leaf area, as well as higher values of TCA and, respectively, of maximum photosynthetic capacity per area ($A_{\max,a}$). It seems that, female trees stronger invest in xylem efficiency and carbon gain, while male trees invest more in defense. The phenotypic plasticity of the sexes in response to sun and shade conditions was approximately the same, with the remark that each sex expresses different optimization strategies.

To our knowledge, in Romania, no studies have been done from this point of view. Therefore, our results hope to bring some data regarding the morphological, micromorphological and physiological traits of the male specimen over time, including preparation for the winter season, in the case of the temperate continental climate specific to our country.

MATERIALS AND METHODS

Biological material

The study has been performed using the collected leaves from the male tree of *Pistacia vera* L., grown as ornamental species in the Botanical Garden of the University of Agronomic Sciences and Veterinary Medicine of Bucharest, Romania (USAMV Bucharest).

The specimen has been brought 35 years ago from Grece to be acclimatized and studied in the Bucharest area (Romania).

The Bucharest city is located at 44°24'49" North latitude and 26°5'48", East longitude, 90 m altitude and temperate-continental climate, with 585 mm/year rainfall and 10.86°C the average of the temperature.

On the active growth season, in June and July months, the leaves of pistachio have been collected from the branches located at the height of 1.5 m within South-West exposure and morphological, micromorphological and anatomical observations were made on the collected leaves.

Micromorphological and anatomical assessment

The micromorphology of the petiole and leaf lamina has been assessed with the scanning electron microscope (SEM) belonging to the laboratory of microscopy and plant anatomy in the USAMV Bucharest research center.

For anatomical analysis have been performed the transversal sections in the leaf petiole and in the leaf lamina and there were clarified with Chloral-hydrate coloured with the Carmine-Alaunate and Iodine Green according to the classic method (Andrei et al., 1975).

Both epidermises of the leaves were collected and used for counting the number of stomata per square millimeter and for measuring the size of stomata (length, width). In the transversal sections of the petiole and leaf lamina has been measured the size of the specific tissues.

The remarks, images and the measurement of the anatomical structures were made with the optical microscope Leica DM1000 LED, video camera Leica DFC295 belonging to the Laboratory of microscopy and plant anatomy of the same research center.

The photos have been taken using the 4X and 20X objectives and the measurements of the tissues were made at the objective of 20X.

For counting of the stomata, the objective 40X has been used. The photos and measurements of the stomata were made at the objective 20X.

Determined physiological indicators

Gas exchange measurements: net photosynthesis (P_n) ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$); transpiration rate (Tr) ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$); stomatal conductance (g_s) ($\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$) and intercellular carbon dioxide concentration (C_i) ($\mu\text{mol CO}_2 \text{ mol}^{-1}$) have been determined during October with the

help of a portable photosynthesis system (LCPro+ Bio-Sciences), between 10:00 am and 12:00 pm, for ten leaves still visibly green, sun-exposed, as well as yellowed leaves. Then, the following physiological indicators were estimated: water use efficiency (WUE) (Pn/E); quantum yield (QY) (ϕCO_2) ($Pn/PPFD$) and Tr/g_s . Total chlorophyll content was estimated by using the chlorophyll meter CCM-300 and results were expressed as mg/dm^2 FW. The study of membrane stability has been carried out by the conductometric method. Thus, electrolytes transport through membranes (MET) ($\mu S\ cm^{-1}g^{-1}$), and total electrolyte content (TE) ($\mu S\ cm^{-1}g^{-1}$) have been determined. Based on them, the synthetic indicator called electrolyte leakage (EL) (%) was calculated. Statistical analysis has been performed by the Student's T-Test.

RESULTS AND DISCUSSION

Leaf morphology and micromorphology

The male tree specimen of *P. vera* from botanical garden is low size (4 m height), with a short trunk and a strongly branched globular crown.

The leaves are alternate, simple and composed of three leaflets, ovate – elliptical, with entire edge, mucronate or obtuse leaf tip, glossy (Figure 1).



Figure 1. *Pistacia vera* L., male specimen from the Botanical Garden, USAMV Bucharest

As results of micromorphological analysis it has been observed that the leaves had non-glandular hairs on the petiole and on both epidermises of leaf lamina, more numerous in the lower

epidermis and the stomata were on both epidermises (Figures 2 a, b, c).

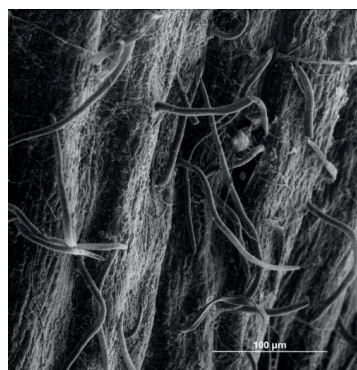


Figure 2 a. Micromorphology of the petiole - *Pistacia vera* L. (SEM 800 X)

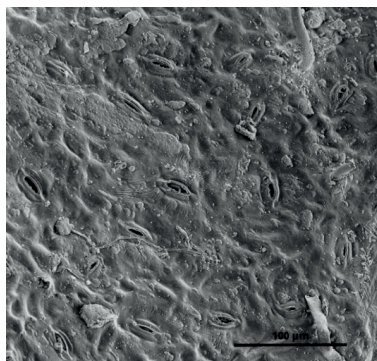


Figure 2 b. Micromorphology of the lamina - analysis of the upper epidermis - *Pistacia vera* L. (SEM 800 X)

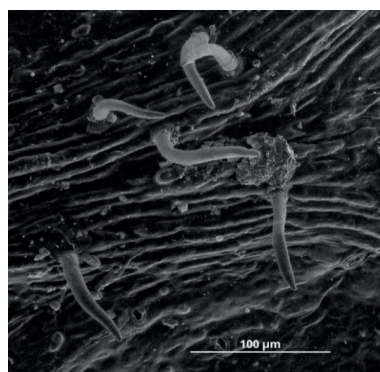


Figure 2 c. Micromorphology of the lamina - analysis of the lower epidermis - *Pistacia vera* L. (SEM 800 X)

Leaf anatomy

The leaf anatomy of the *P. vera*, analyzed male tree is in line with the data from literature (AL-Saghir et al., 2006).

Petiole anatomy

In the transversal section, the petiole is ovoidal with two small lateral prominence in the adaxial zone (Figure 3 a).

From the border to the center of the petiole there are the following tissues: epidermis, collenchyma, parenchyma, vascular bundles and the pith (Figure 3 b).

The epidermis is uniseriate with the medium thickness of 12.4 μm covered by a thick cuticle of 3 μm .

In the epidermis there were observed the stomata and the unicellular non glandular hairs.

The angular collenchyma is 71.2 μm thick.

In the adaxial collenchyma there were identified the chlorenchyma zones.

The parenchyma is formed by ovoidal cells with the thin walls and intracell spaces, having 53.5 μm width average. In the parenchymatic cells there were identified the mineral crystals.

The vascular bundles are collateral-opened, with secondary structure generated by the cambium and bordered by the sclerenchyma and separated by the principal medullar rays.

In the abaxial zone there were identified 6-9 semicircle big vascular bundles and in the adaxial zone were 4-5 horizontal small vascular bundles.

In the phloem, the secretory ducts were identified, having a large lumen with 88 μm .

The thickness of the phloem has been 68 μm and the xylem has been 105 μm .

The pith is star shape with parenchymatic origins and 42 μm size with mineral crystals in the cells.

The micromorphology and the size of the leaf tissues can be significant influenced by the climatic conditions and air pollution with the cars gas emissions (Amara, 2017; Nadjat et al., 2020; Açar, 2023).

Lamina anatomy

The front view of the epidermises presented the polygonal cells with the thin walls (Figures 4 a and b).

The leaf is amphistomatic and the stomata are anomocytic type, like in the speciality literature (AL-Saghir & Porter, 2005).

In the upper epidermis, the average of the stomata density is 105 per mm^2 , and 145 per mm^2 in the lower epidermis.



Figure 3 a. Transversal section in the petiole - *Pistacia vera* L. ad.ep - adaxial epidermis; ngh - non glandular hairs; co - collenchyma; pa - parenchyma; sc - sclerenchyma; ph - phloem; xy - xylem; sd - secretory duct; pi - pith; ab.ep - abaxial epidermis (objective 4X)

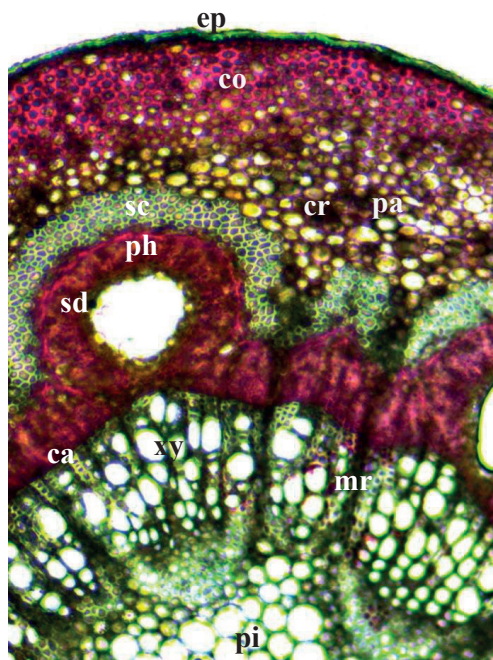


Figure 3b. Transversal section in the petiole (detail) - *Pistacia vera* L.

ep - epidermis; co - collenchyma; cr - crystal
pa - parenchyma; sc - sclerenchyma; ph - phloem;
ca - cambium; xy - xylem; sd - secretory duct;
mr - medullar rays; pi - pith (objective 20 X)

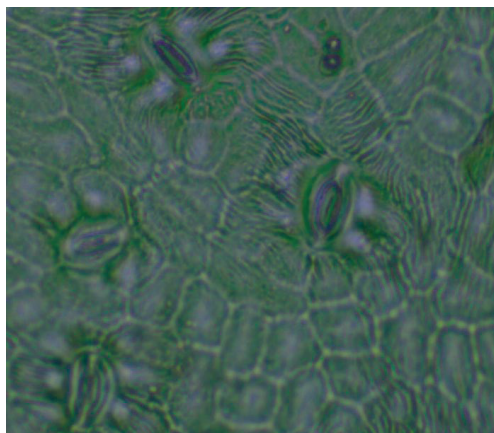


Figure 4 a. Analysis of the upper epidermis of the lamina - *Pistacia vera* L. (objective 20X)

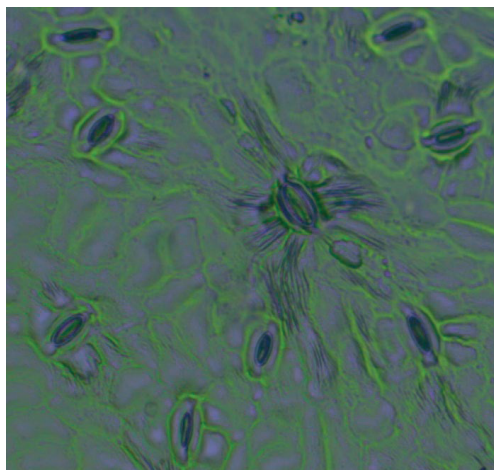


Figure 4 b. Analysis of the lower epidermis of the lamina - *Pistacia vera* L. (objective 20 X)

The average size of the stomata is: 19 μm length x 16 μm width for upper epidermis and 22 μm length x 17 μm width for lower epidermis. The stomatal density is significantly influenced by the amount of precipitation of the geographical area (AL-Saghir, 2006). The highest stomatal density and the lowest stomatal length and width were obtained under severe hydric stress (Arzani et al., 2013). In cross-section through the lamina the epidermises are uniseriate with an average thickness of 16 μm (Figure 5). The leaf mesophyll is equifacial (bilateral), with palisade tissue under the both epidermises and spongy tissue in the middle.

There are structural differences in the mesophyll of the leaves between different species of *Pistacia* (Leng et al., 2007; Mehdeb et al., 2016). The palisade tissue below the upper epidermis consists of 2 rows of elongated cells, rich in chloroplasts, with an average thickness of 70 μm .

The palisade tissue located under the lower epidermis consists of 1-2 rows of short cells with an average thickness of 35 μm .

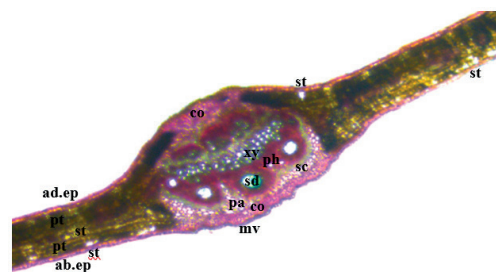


Figure 5. Transversal section in the lamina - *Pistacia vera* L. ad.ep - adaxial epidermis; pt - palisade tissue; st - spongy tissue; st - stomata; mv - middle vein; co - collenchyma; pa - parenchyma; sc - sclerenchyma; ph - phloem; xy - xylem; sd - secretory duct; ab.ep - abaxial epidermis (objective 4X)

The spongy tissue is compressed, consisting of 3-4 rows of ovoid cells, with a lower content in chloroplasts and the average of thickness 32 μm . In the mesophyll there were identified the collateral bundles with the xylem to the adaxial epidermis and phloem to the abaxial epidermis. In the middle vein of the leaf is ellipsoidal shape with a uniseriate epidermis under which an angular collenchyma has been observed, more developed in the adaxial part and a parenchyma. In the middle vein were revealed 3-4 collateral vascular bundles, 3 smaller vascular bundles located in the adaxial part and 4 bigger vascular bundles towards the abaxial part. In the phloem of the vascular bundles from the abaxial part has been identified the secretory ducts, with a diameter of 45 μm .

Leaf physiology

The obtained results can be seen in Table 1. With the exception of Tr/g_s (p value = 0.380635), statistically significant differences ($p < 0.05$) were recorded between the still visibly green leaves and the yellowed ones in a proportion of about 75% (yellowed - red) for all the others. Very significant higher values have been obtained for yellowed leaves (Pn , WUE , QY , MET and percentage EL), while lower data

can be seen for Tr, g_s , Ci, and TE, as against to the still green leaves.

The explanation could be that during the autumn period, the establishment of senescence of the leaves is a process that takes place gradually and depends largely on the climatic conditions of the respective year, especially those related to the level of day-night temperature variation, the presence or absence of precipitation, the degree of drought etc. For the visibly green leaves, it is possible that the reduced values of Pn (and of the associated indicators), are due to the intensification of the respiration process, the rate of which exceeds the rate of Pn. Regarding the transpiration rate, the low values for leaves in an advanced degree of yellowing may be due to the drastic decrease in leaf turgor (implicitly of the stomata guard cells) and as a result the stomata close. This behavior is also supported by the reduced values of g_s (1.62 times lower values for YL vs. GL).

As for the membrane stability, the significant differences are noted from a statistical point of view for each individual indicator, but it is worth noting that in the case of yellowed leaves, the EL

(%) was consistently and significantly higher, almost double compared to green leaves. This behavior is in close relation with the reduction of the degree of selectivity of the membranes together with the installation of senescence of the leaves during the fall. Furthermore, it is interesting that TE content was greatly reduced in the yellowed leaves (about twice), which indicates a redirection of the mineral ions from the leaves to other tissues of the plant, before the leaves fall. It is a normal physiological process of preparing the plants for entering the dormant period and ensuring survival for the next season. An increased value of EL (%) was also recorded in the case of the incident of different stress factors, such as low temperatures in the case of a cold climate. The studies carried out by Ravari et al. (2023) on different pistachio genotypes to select tolerant varieties in such areas, highlighted the specific variability depending on the genotype. Thus, at a temperature of -16 C, for *P. atlantica* and *P. khinjuk* the EL values were the highest, while for *P. terebinthus* and *P. vera* var. Sarakhs there were determined the lowest values for this indicator.

Table 1. Physiological indicators in the leaves of *Pistacia vera* L., in the autumn season

Variable	Green Leaf (GL)	Yellowed Leaf (YL)	the <i>p</i> value	Statistical significance (YL versus GL)
(Pn) (net photosynthesis - $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	-0.33 ± 0.10	0.27 ± 0.15	0.006015	**
(Tr) (transpiration rate - $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$)	1.29 ± 0.15	0.84 ± 0.07	0.004894	00
(g_s) (stomatal conductance – $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)	0.026 ± 0.005	0.016 ± 0.002	0.017055	0
Intercellular carbon dioxide concentration (Ci) ($\mu\text{mol CO}_2 \text{ mol}^{-1}$)	418 ± 11.85	390.60 ± 9.81	0.009381	00
Water use efficiency (WUE)(Pn/Tr) ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} / \text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$)	-0.286 ± 0.17	0.2268 ± 0.28	0.006431	**
Quantum yield (QY)(ϕCO_2) (Pn/PPFD) ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1} / \mu\text{mol photons m}^{-2} \text{ s}^{-1}$)	-0.00032 ± 0.000179	0.00018 ± 0.000218	0.004423	**
Tr/ g_s ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1} / \text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$)	53.82 ± 9.71	55.70 ± 8.63	0.380635	NS
Total estimated chlorophyll content (mg dm^{-2})	6.468 ± 0.125	5.676 ± 0.159	0.005013	00
Electrolytes transport through membranes (MET) ($\mu\text{S cm}^{-1} \text{ g}^{-1}$)	904.50 ± 3.88	1275.20 ± 16.64	0.010951	**
Total electrolyte content (TE) ($\mu\text{S cm}^{-1} \text{ g}^{-1}$)	2031.11 ± 7.97	1986.90 ± 4.75	0.023145	0
Electrolyte leakage (EL) (%)	44.53 ± 0.02	64.18 ± 0.68	0.010814	**

Results are shown as mean values \pm standar error (SE) (n = 5). The comparison was done by the paired T-Test, in Excel, by comparing the values of GL and YL; $p \geq 0.05$: no statistically significant differences (NS); $p \leq 0.05$: statistically significant difference at 95% confidence level (*); $p \leq 0.01$: statistically significant difference at 99% confidence level (**); $p \leq 0.001$: statistically significant difference at 99.99 % confidence level (***)

In the specialized literature, we have not found comparative studies (still green leaves and yellowed leaves) focused on the behavior of the *P. vera* species during the establishment of leaf senescence. Research has focused on the

dynamics of some physiological indicators during the phenological phases (Marino et al., 2018 a), but more specifically the impact of some stress factors on plant morphology and physiology (Mehdi et al., 2011; Esmaeilpour et

al., 2016a; Ben Hamed et al., 2023; Ravari et al., 2023). In addition, the possibilities of counteracting the negative effects of the external factors (Marino et al., 2018b; Todros et al., 2021; Marino et al., 2023; Miri et al., 2023), as well as intrinsic ones (e.g., sink-source balance) (Marino et al., 2023) expressed through morphological and physiological changes, have been addressed.

The maximum net photosynthetic capacity (P_{nmax}), apparent quantum yield ($\mu\text{mol m}^{-2} \text{s}^{-1}$) and, dark respiration ($\mu\text{mol m}^{-2} \text{s}^{-1}$) change drastically between leaf phenological stages (Marino et al., 2018). It turned out to present a similar evolution to that specific to other plant species in general. The P_{nmax} values were low in young leaves (15.09), while the respiration rate showed higher values (2.86), following that once the maturity phase is reached, the assimilation of carbon registers maximum values (19.39), and the rate of the respiration process decreases (1.45). With the onset of senescence, the efficiency of photosynthesis was markedly reduced (12.78).

Marino et al. (2023) highlights the importance of the source-sink balance, because otherwise when the activity at the sink level is higher, the photosynthesis rate will also be at higher values. However, this can accelerate the leaf senescence, simultaneously with the decrease in the photosynthesis process, which at the end of the vegetation period, for deciduous species, can mean an insufficient accumulation of the reserve compounds, to successfully survive during dormancy and the start of vegetation in the following year.

The comparative studies carried out on *P. vera* and *P. atlantica* exposed to drought and later rehydration highlighted an evident reduction of gas exchange and chlorophyll content in *P. vera*, which indicates a lower degree of its adaptation to drought. At the same time, even if the hydric status after hydration was restored in both species, the physiological indicators such as, net photosynthesis, transpiration rate, and stomatal conductance had lower values than those determined in the well-watered seedlings (Ben Hamed et al., 2023).

To successfully overcome the drought periods, different procedures have been addressed by researchers. Todros et al. (2021) tested different combined water harvesting techniques

(mulching \times micro-catchment) for *P. vera* in Northern Jordan and found a significant improvement in soil characteristics (e.g., water content) as well as morphological indicators (e.g., plant height, number of leaves on the tree) and respectively, physiological ones.

Furthermore, the behavior of *P. atlantica* and *P. vera* seedlings under conditions of water stress, excess light and temperature proved to be different in terms of relative water content, net assimilation, transpiration, P_n/g_s and P_n/C_i (Ben Hamed et al. 2021). For all these physiological indicators, the values were higher in *P. atlantica*, compared to *P. vera*, and the authors explanation refers to the activation of some internal mechanisms for efficient osmotic adjustment, as well as photoprotection at the level of the photosystem II.

Although pistachio is known to be resistant to soil drought, however *P. vera* cultivars have a different physiological behavior, as highlighted by the studies carried out in the greenhouse by Esmaeilpour et al. (2016 a), by applying two levels of osmotic stress ($\Psi_s = -0.75$ and $\Psi_s = -1.5$ MPa). In such conditions, the relative water content and the water potential of the leaves decreased significantly compared to the control, while the water use efficiency had higher values, for all the analyzed varieties. No significant differences were recorded regarding the stomata density and their dimensions, even if 'Ohadi' and 'Kaleghochi' had the widest stomata, whereas 'Akbari' had the most stomata number. With good reason, the authors specify the need to carry out some studies in field conditions, to elucidate these aspects.

Also, the studies carried out by Mehdi et al. (2011) in 5-year-old female trees (Mateur variety grafted on *P. vera*) by applying 3 levels of salt stress, highlighted the fact that in the case of the medium salinity level (5 dS/m EC_w) the growth in length and number of axillary shoots was stimulated. On the other hand, at the highest used electrical conductivity (EC) value (12 dS/m), the growth indicators decreased significantly, and the content of proline and soluble carbohydrates in the leaves significantly increased. However, no significant differences were recorded relative to the leaf's electrolyte leakage.

The studies carried out by Ravari et al. (2023) on 9 *Pistacia* genotypes under the action of

negative temperatures highlighted the variability of the determined indicators (electrolyte leakage percentage, soluble carbohydrates, phenolic compounds) in relation to the species, but what should be highlighted is the fact that *P. vera* var. *Sarakhs* was on his best behavior.

CONCLUSIONS

The male specimen of *Pistacia vera* L., grown in the Botanical Garden of USAMV Bucharest is a small tree, with simple but also trifoliate leaves.

In the petiole of the leaves and in the epidermis has been observed the unicellular non-glandular hairs.

The vascular bundles in the petiole and median vein are of the collateral type, with secondary structure.

Secretory ducts are present in the phloem.

The leaf is amphistomatic, with stomata of the anomocytic type.

The mesophyll of the leaf is equifacial.

During the autumn senescence, there are major changes in the physiological processes of the leaves, closely interdependent with the growing climatic conditions (characteristic of each year), so that the plants can successfully cross the dormant period and be able to successfully start a new growing season.

Physiological indicators can be useful for the characterization of the pistachio adaptation mechanisms in the resilience integrated framework.

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