Micro-Morphological, Anatomical and Statistical Correlative Evaluation Between Different Altitudes and Leaf Structural Features of *Nerium oleander* L. (Apocynaceae), Growing in the Middle-West Taurus, Turkey

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Key words: *Nerium oleander*, altitude, leaf-anatomy, micro-morphology, statistics

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Abstract: *Nerium oleander* (Apocynaceae) which spread over a wide area along the Mediterranean coast, is an important indicator plant of Mediterranean elements. The objective of the current research was to reveal the altitude effect of *N. oleander* in the Middle-West Taurus region situated in Turkey. In this study, it is statistically evaluated that the micro morphological and anatomical changes observed in tissues of the leaves collected at different altitudes. Leaf structure of *N. oleander* demonstrated morphological, epidermal and anatomical alterations at various elevations. Statistical analysis was subjected to analysis of covariance (ANCOVA). According to the Levene test results, it is determined that the Wmed and Lmed averages of some anatomical parts such as spongy parenchyma cells, collenchyma and xylem layers, increase as the altitude escalates. This result has been supported by the correlative effect of locality and anatomical parts and it is significantly relating the measurement of Wmed ($p = 0.000<0.05$). In micro morphological studies, trichome density and cavities were seen in the stomatal crypt chamber, the sizes of the upper and lower epidermal cells and the structures of anticlinal walls were examined in the superficial incisions. Considering the altitudes starting from 3 m up to 898 m, it is observed that the trichomeson the leaf epidermal surface were observed to decrease as altitude increases, while any remarkable difference was not found for the trichomes located in the stomatal crypt. Notwithstanding, the crystals in the leaf mesophyll layer were more concentrated at low altitude (3 m) while abundance decreased at higher altitudes (898 m).

INTRODUCTION

The Apocynaceae is a quite large angiosperm family about 155 genera and 2000 species distributed primarily in the tropics and subtropics, poorly represented in the temperate regions[1]. The family is represented by 11 genera and 28 taxa in Turkey. As one of the most important genera of the family, *Nerium* is characterized by only one species "*Nerium oleander* L." in the world. This species, known as "zakkum" is spread over a wide area along the Mediterranean coast in Turkey and it is naturally seen especially among shrubs in the creeks and rivers[2]. It is also called "acıhayıt", "delihayıt", "acıağaç", "ağı", "develik" and "patlangeç" due to its harsh taste. Its fresh latex is used externally against scorpion stings and snake bites as an analgesic antidote in the West and South Anatolia[3-5].

Morphological and anatomical properties of plants also affiliated with abiotic factors[6]. The stomata of leaves are very prominent physiological devices for photosynthesis and transpiration in vascular plants[7]. Yet, it is not known whether these particular responses of leaf morphology to temperature, extend to other features of climate that also show a variation with altitude[8].

Altitude takes effect on plant species and vegetation, indirectly. As the altitude increases; temperature, relative humidity and water vapor are decreased. While the altitude increases, it is known that the temperature has been falling, the ground water supply has been becoming more difficult and the vegetation period has been reduced[9]. It is quite limited that the studies conducted on revealing what changes about micro-morphological and anatomical characters depending on altitudes.

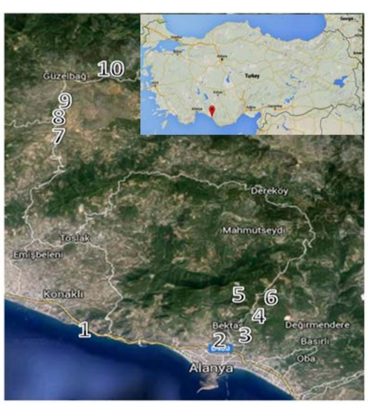
Notwithstanding, it is not encountered in any detailed studies about the existence of the kind of the morphological and anatomical changes on the level of plant tissue or cell, except some studies[10, 11, 7]. *Nerium oleander* which spread over a wide area along the Mediterranean coast is an important indicator plant of Mediterranean elements. The first reason we chose Oleander in this study is its successful adaptation to the climate of the Middle Taurus, located in the Mediterranean region and showing a good growth even in different habitats. The second; this species can assert itself to be spread out easily at a large elevation scale starting from sea level to about 1000 m.

Nerium oleander, we discuss in this study is a kind of typical elements of Mediterranean flora. The Mediterranean Region of Turkey has been deemed appropriate in terms of the collection of species and the reliability of the study since it is one of the best regions for monitoring Mediterranean elements. This is the first study to reveal anatomical changes expressed by *N. oleander* along with an altitudinal gradient.

MATERIALS AND METHODS

The study areas: The leaves of *N. oleander* specimens which naturally distributed in and surroundings of Alanya (Antalya-Turkey) constitute the main material of the study. This area is located between latitude 36.773357 N and longitude 32.097221 E (Fig. 1). The main climatic data of different altitudes in the study area is given in Table 1. The average annual wind speed in the region is 1.6 m/sec according to the Meteorological Observation Station records. This value is 1.9 m/sec in January and February which have the highest monthly average of the windspeed while it is 1.4 m/sec in May and August which have the lowest value, respectively[12].

Plant material and sampling: The localities and altitudes that the leaves were collected, as well as various climatic data are presented in Table 1. During the study period, also after detection of plants spread over ten different localities, the leaves which collected on the same day were stored in appropriate conditions by putting into labelled-glass-lid bottles containing 70% alcohol for the anatomical studies and in terms of the reliability of the statistical data. Biometric and morphometric measurements on 10 mature leaves were taken from the South-facing parts of 5-10 years old plants growing in open areas in each altitude (Table 2). In the field, the



Loc #	Altitude (m)	Latitude	Longitude
1	3	36.56842523	31.91165954
2	60	36.55788398	32.00097039
3	149	36.55795724	32.0096983
4	240	36.56030345	32.008317
5	376	36.57357361	32.0081212
6	474	36.56760232	32.0158406
7	495	36.68976658	31.8932408
8	719	36.69932421	31.8911004
9	778	36.71167597	31.89663112
10	898	36.73161007	31.92567945

Fig. 1: The geographical location of the study area (Alanya/Antalya)

Table 1: Nerium oleander localities, altitudes and climatic data where they were collected

Locality (#)	Altitude (m)	Temperature (°C)* Max (mean)	Min (mean)	Relative humidity (%)	Rainfall average (mm) relative humidity (%) Max (mean)	Min (mean)	Atmospheric pressure (mean)	Location
1	3	29.3	17.6	61.8	168.6	31.1	1012.1	Konaklihalkplaji
2	60	29.3	17.6	61.8	168.6	31.1	1012.1	Yaylayoluçikisi
3	149	28.4	15.3	61.0	275.0	38.6	1012.1	Dorukevleri
4	240	26.1	14.0	61.0	743.0	59.4	1012.1	SeyirTepesi
5	376	24.7	13.8	62.0	1002.0	83.9	1012.1	Tepemah.
6	474	23.6	12.6	62.1	1087.0	91.3	1012.0	Snowdrop Rest.
7	495	23.6	12.6	62.1	1087.0	91.3	1012.0	Sogukpinar
8	719	20.3	10.0	63.0	1090.0	92.8	1008.3	Gözübüyük
9	778	19.8	9.0	63.0	1092.0	93.7	1007.9	Beyobasi
10	898	18.7	8.1	64.0	1095.6	95.2	1006.3**	Güzelbagçikisi

*Temperature (maximum and minimum means of centigrade), average rainfall (millimeters), relative humidity(percentage). **The above data are the average value of the last 15 years

Table 2: Measurement data showing the change of anatomical structures according to the altitudes

Loc#	Altitude (m)	Leaf width (L_w) (cm)	Leaf length (L_l) (cm)	Stomatal density* Pore/mm2 (mean±SD)	Stomatal index	No. of epidermal cells (nEC)	PCI	Guard cell length* (µm) mean±SD
1	3	2.76	15.81	0.55±4.422	2.636	886.35	0.022	20.16±0.525
2	60	2.83	19.28	0.44±3.348	2.554	919.35	0.018	20.03±0.462
3	149	3.23	16.40	0.44±4.035	2.484	922.55	0.018	20.07±0.607
4	240	3.35	19.88	0.37±3.604	2.379	1021.9	0.015	19.88±0.487
5	376	3.14	17.59	0.45±4.163	2.347	1040.1	0.018	20.02±0.619
6	474	3.02	17.83	0.47±3.308	2.373	1049	0.019	19.18±0.362
7	495	3.07	17.91	0.43±3.683	2.357	981.7	0.017	19.39±0.446
8	719	3.13	17.91	0.43±6.935	2.374	982.95	0.017	19.60±0.684
9	778	3.18	17.95	0.44±4.795	2.170	1122.65	0.017	19.05±0.511
10	898	3.23	19.15	0.40±3.972	2.058	1189.8	0.016	18.82±0.438

*Stomatal density and guard cell length (data are mean of 15 replicates and standard deviation); Ll: Leaf length; PCI: Potential conduct index; SD: Standard deviation

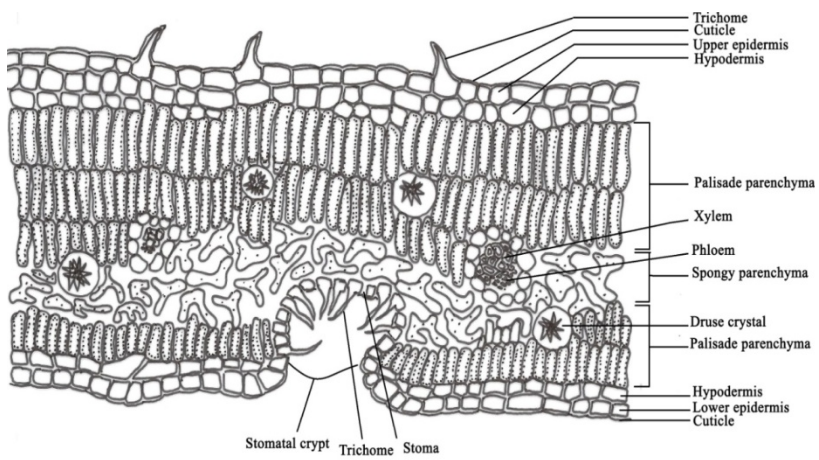


Fig. 2: The illustration of the leaf mesophyll layers. Photo credited to Selami Selvi

obtained plant samples for anatomical review were fixed in 37% FAA (Formaldehyde-Acetic acid-Ethanol) solution for 24 h and then preserved in 70% alcohol.

Leaf anatomy analysis: Anatomical data were obtained by computing the averages of the measurements on 10 cross-sections and 10 surface-cuts taken by hands from each lamina and only cross-sections of each petiole part of each leaf. Obeying Vardar method, the sections placed on slides were stained with phloroglucinol HCl solutions, then chlorophyll in leaves was removed with chloral hydrate before treating with glycerine-gelatine to make permanent slides. Sections were examined with a Nikon Eclipse E200 light microscope attachment camera and transferred to digital media by taking their photographs. Biometric measurements on the sections were conducted via the Toup Cam Software program. The anatomical structure of *N. oleander* leaf portions is shown by drawing (Fig. 2). The micro-morphological characters such as stomatal crypt trichome, stomatal crypt chamber's; upper and lower epidermal cell dimension and epidermis anticlinal wall structure were examined.

Statistical analysis: In this study, it was found appropriate to use the ANCOVA in 10 different regions that Wmed (the leaf width medium) and Lmed (the length medium) variables examined. Literally, one-factor ANCOVA is often used in an experimental design that some different subject groups were compared with their values for a dependent variable and associated with constantly changing one or more dependent variables. However, the effect of the process can be examined by this method in a pattern that concerned with a group of two or more subjects fully randomized by controlling continuous measurements belonging to an external variable related to the dependent variant with the values for the dependent variables of subjects[13]. Data were subjected to analysis of variance conducted by using SPSS 20 statistical program. Then, the data were tested by Levene's method and the significant difference was found at $p<0.05$.

RESULTS

Micromorphological and anatomical analysis: The mesophyll cells of leaves taken from different altitudes are shown in Fig. 3. Considering this figure; there was no remarkable difference of altitude ranging from 898-3 m for the leaf mesophyll thickness. However, it was observed that stomatal crypts wee more frequent and intense at low altitudes while more infrequent at high levels. Similarly, the trichome structures were found more concentrated in low altitudes while it was seen that the number decreased towards higher elevations (898 m). *N. oleander* is a xeromorphic species and its stomata are hidden in the lower surface of the leaves. Stomatal crypts were large chambers in the mesophyll, covered with an epidermis containing stomata as well as trichomes (hairs) that projected into the crypt (Fig. 2, 5d). In the leaf anatomical structure of Oleander, Druse crystals were observed (Fig. 2, 3). The crystals in the leaf mesophyll layer were more concentrated at low altitude (3 m) while abundance decreased at higher altitudes (898 m) (Fig. 3).

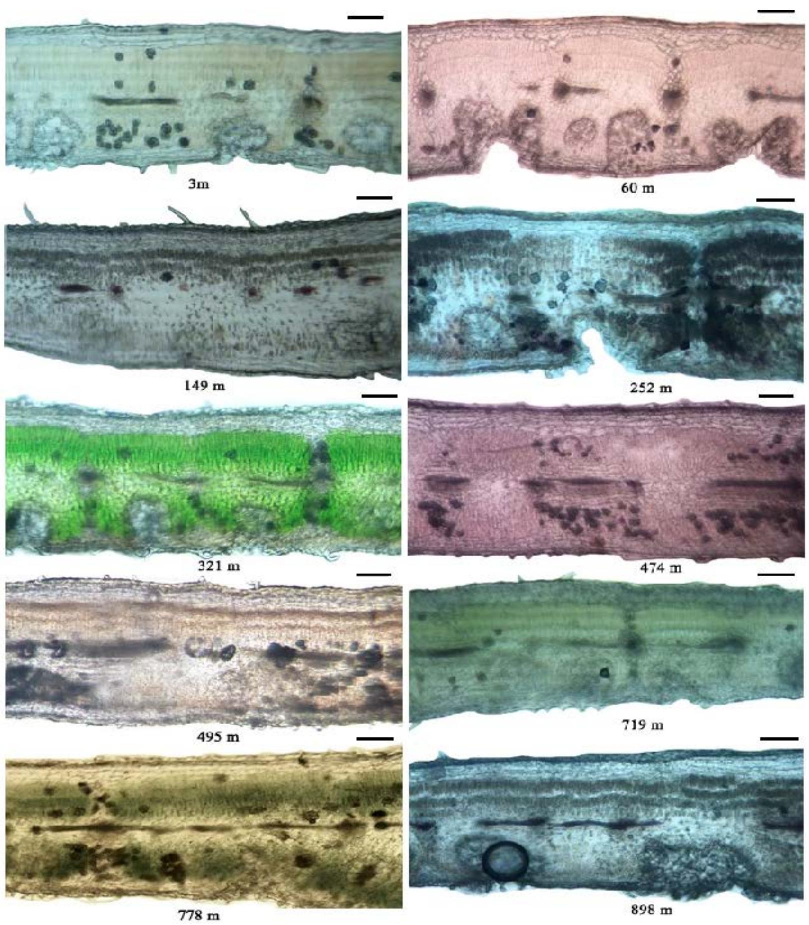


Fig. 3: Comparative cross-section of the mesophyll layer of leaves taken from different altitudes (Scale bar: 100 µm). All photos by the author

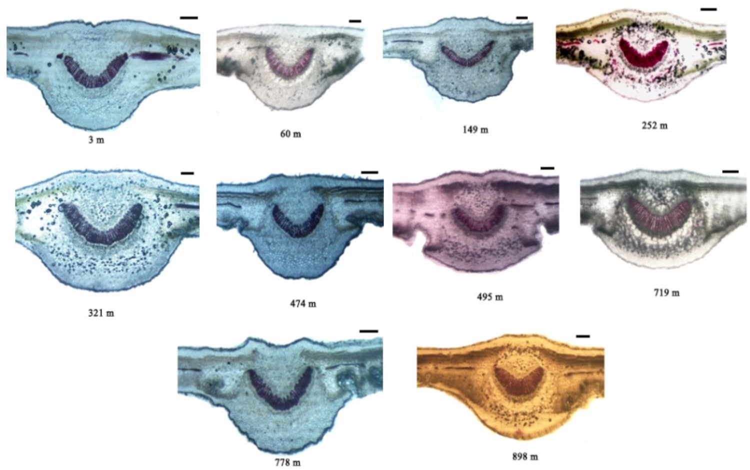


Fig. 4: The comparative cross-sections of the petioles taken from different altitudes (Scale bar: 200 µm)

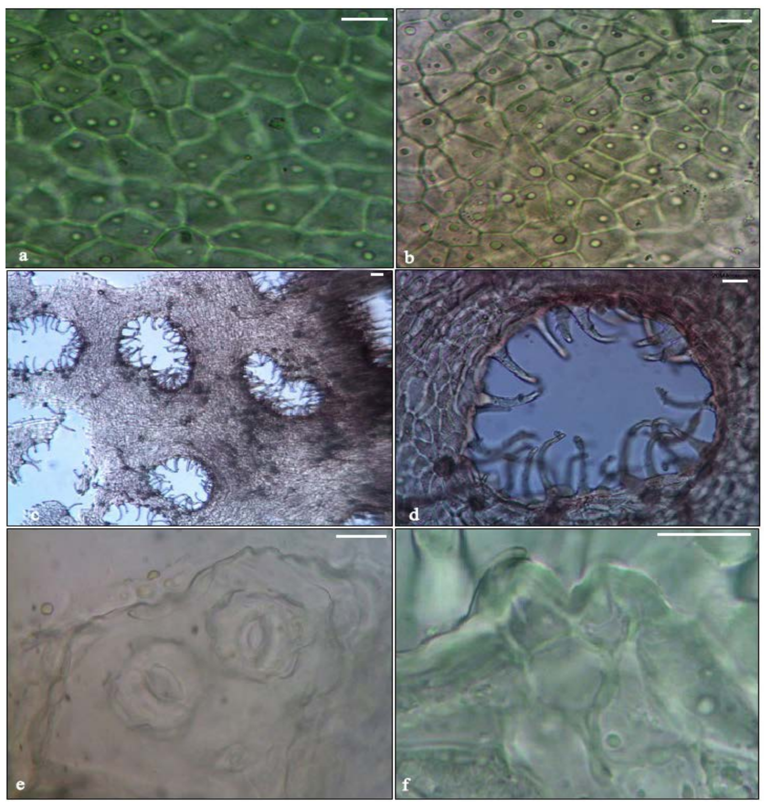


Fig. 5: The leaf epidermal surface images of *N. oleander* (a) Abaxial surface (149 m), (b) Adaxial surface (474 m), (c) Stomatal crypts on abaxial surface (719 m), (d) Stomatal crypt (778 m), (e) Stomata view of the surface sections (50 m), (f) Stomata view in cross-section (50 m) (Scale bar : 20 µm)

The crescent-shaped (abaxial bundle) structure of the midvein in the petiole was broad in low altitudes, whereas the angle narrowed at increasing altitudes. Again, the projections on the abaxial surface were found to be more pronounced and protuberant at low altitudes (Fig. 4). There were also some significant differences in the width and length of cells and tissues comparing with the altitudes in terms of the statistics (Table 3, 4 and Fig. 5-7). Throughout the micromorphological analysis, the anticlinal walls of leaf epidermal (adaxial and abaxial

Table 3: The Levene's test results for Wmed (Leaf width medium)

Source	Type III sum of squares	df	Mean square	F	Sig.
Corrected model	7104935.002[a]	249	28533.876	78.689	0.000
Intercept	3964452.580	1	3964452.580	10932.944	0.000
Locality	24638.679	9	2737.631	7.550	0.000
Anatomical_parts	6750830.158	24	281284.590	775.711	0.000
Locality * Anatomical_parts	337845.996	216	1564.102	4.313	0.000
Error	36261.528	100	362.615		
Total	9982482.010	350			
Corrected total	7141196.530	349			

α = Reliability; df = Degree of freedom; F = Value of the F (ANCOVA) test; N = Number of the participants; p = Significance; Sig = Level of Significance; Dependent Variable: Width_med. [a]$R^2 = 0.995$ (Adjusted $R^2 = 0.982$)

Table 4: The Levene's test for the Lmed (Leaf length medium)

Source	Type III Sum of squares	df	Mean square	F	Sig.
Corrected model	36437.368[a]	99	368.054	7.919	.000
Intercept	143267.102	1	143267.102	3082.414	.000
Locality	1437.015	9	159.668	3.435	.002
Anatomical_parts	25407.697	9	2823.077	60.739	.000
Locality * Anatomical_parts	10308.779	81	127.269	2.738	.000
Error	2788.732	60	46.479		
Total	177759.000	160			
Corrected total	39226.100	159			

α = Reliability; df = Degree of freedom; F = Value of the F (ANCOVA) test; N = Number of the participants; p = Significance; Sig = Level of Significance; Dependent Variable: Length_med. [a]$R^2 = 0.929$ (Adjusted $R^2 = 0.812$)

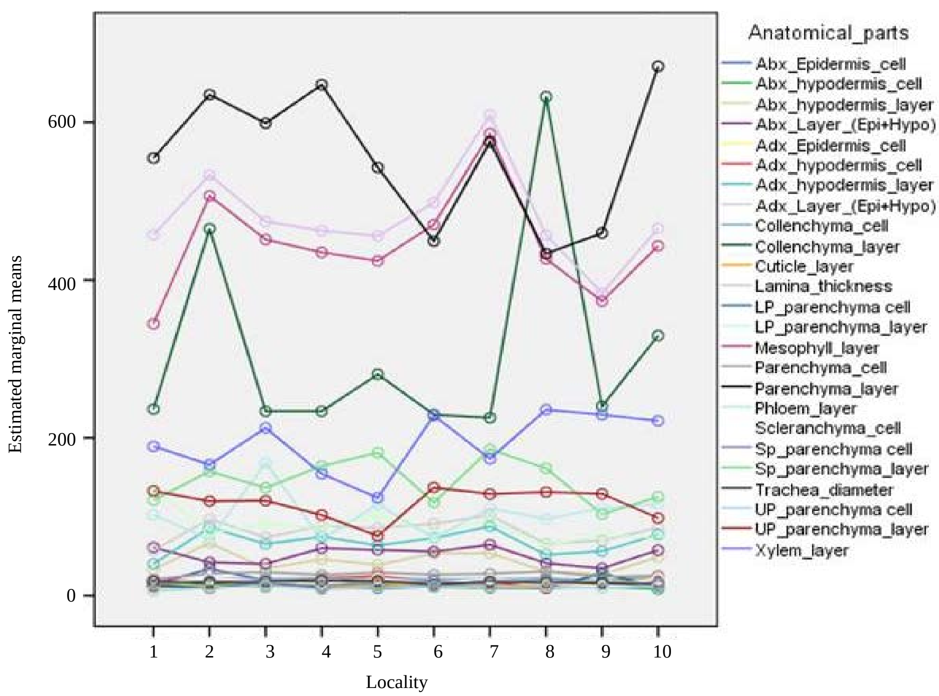


Fig. 6: Estimated marginal means of Leaf Width (Wmed) according to the anatomical parts

surface) cells and trichome density were investigated (Fig. 5). According to the findings on the samples taken from various altitudes starting from 3 m up to 898 m, it was observed that the anticlinal walls of the epidermis cells appeared to be smooth and waveless structures and did not exhibit any remarkable variability depending on the elevation (Fig. 5). The trichomeson the leaf epidermal surface were observed to decrease as the altitude increased, while any remarkable difference was not found for the trichomes located in the stomatal crypt.

Statistical analysis: Examining Table 3, it is seen that the correlative effect of locality and anatomical parts is significant relating to the measurement of Wmed ($p = 0.000<0.05$). This finding means that it is not equal to the calculated regression lines of the anatomical parts

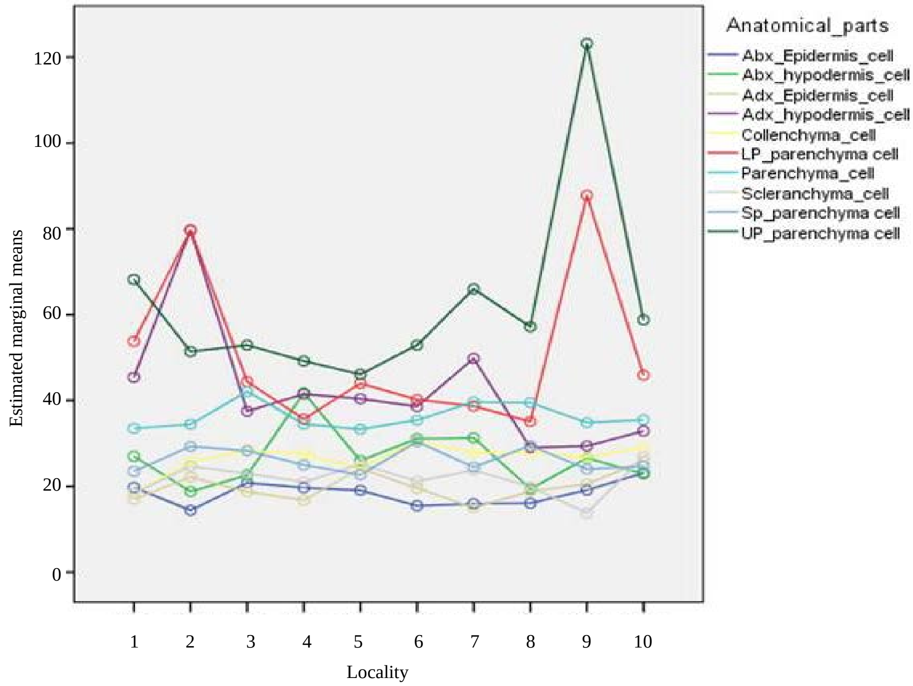


Fig. 7: Estimated marginal means of Leaf Length (Lmed) according to the anatomical parts

of the plants growing at 10 different localities related to the average of Wmed. Levene's test was conducted in order to determine which differences were among the locality groups. As reported by this test, there was a significant difference between the Wmed averages of the plants growing in the 1st locality and the 2nd, 7th, 8th, 10th localities; among the 2nd locality with 4th, 5th, 6th and between in the 3rd and the 9th localities (Fig. 6).

Analyzing Table 4, it is observed that the reciprocal effect of the locality and the anatomical parts are meaningful concerning the evaluation of Lmed ($p = 0.000<0.05$). This data expresses that it is not proportionate on the computed regression lines of the anatomical structures of the plants growing in ten different localities regarding the average of Lmed. As stated in the Levene Test results performed to determine the differences occur between which groups, there are significant differences amid the 1st locality and 9th; between 2nd and 8th and the 3rd among 4th, 5th, 6th, 7th and 8th localities (Fig. 7).

DISCUSSION

*Nerium oleander*is a common fast growing evergreen sclerophyllous, naturally distributed and widely cultivated shrub in the Mediterranean area[14, 15]. The earlier studies[16, 17] have carried out the leaf-micro-anatomical studies, depending on elevation with the species *Pinus roxburghii* and *Origanum vulgare*. Nonetheless, Tiwari *et al.*[7] were describing mainly the relationship between the stomata and the elevation while Kofidis *et al.*[10] explored the changes in the rate of photosynthesis depending on the amount of chlorophyll in leaves at different seasons. In our study, we focused on changes in cell size along an altitudinal gradient from sea level to 900 m. In addition, the densities of trichomes and crystals, located in lamina, petiole and stomatal crypt in the leaves were also examined. The stomata and the guard cells could have been counted and the values such as stomal index and Potential Conductivity Index (PCI) were calculated (Table 2), although, the stomata were observed in epidermal depressions, called "crypts" as Oguchi *et al.*[18] pointed out. This leaf structure of Oleander is a classic example of xeromorphic anatomical adaptation which is believed to be partly responsible for the known drought resistance. As Pagen[19] and Kumar *et al.*[20] mentioned in their studies, we have monitored the oleander leaves which were like a leathery, have been covered with a thick cuticle and stomata found on the lower surface of the leaves and immersed in depressions covered with microscopic hairs to prevent water loss. Likewise, Tiwari*et al.*[7] determined those values and found significant altitude effects in their epidermal investigations on pine leaves. For why, crystals are important and unchanging characters in the systemic terms[16, 17, 21, 22]. The anticlinal structure of the wall is also important in terms of taxonomic diversity. Metcalfe and Chalk[16] and Decraene *et al.*[23] confirm that this characteristic can be used for taxa in generic or specific level.

The fact that no significant difference was found in the correlation between altitude and some anatomic structures such as leaf mesophyll thickness and the surface structure of the anticlinal walls of the epidermis cells (as to be smooth or waved) was found to be an unexpected negative result. At least, the difference in cuticle layer thickness would have been expected but because of the fact that the plant did not propagate at higher altitudes due to being a Mediterranean element, or just sampling of individuals raised in high shade media, any significant difference could be detected. However, the presence of the stomatal crypts, trichome structures and the crystals in the leaf mesophyll layer have been more frequent and intense at low altitudes while more infrequent at high levels as a promising positive result for future studies. Also, the results of being crescent-shaped (abaxial bundle) structure of the midvein in the petiole has been broad in low altitudes whereas the angle narrows at increasing altitudes and the existence of the projections on the abaxial surface as to be more pronounced and protuberant at low altitudes can be considered important data for current or prospective oleander producers and researchers.

One-factor ANCOVA analysis was conducted in order to determine whether there was any significant difference among the leaf width medium (Wmed) and the length medium (Lmed) values according to the localities and anatomical part data. The Levene's test was performed to identify among which groups this difference occured. The obtained data is consistent with the correlation between the anatomical parts and the altitude. Yet, it is unfortunate that the number of individuals to take samples decreased as the altitude rose, considerably and samples were taken from individuals which were found to be single specimens and showed growth in a shady environment, compulsorily. Thus, the integrity of meaning may have been disrupted by individuals raised in shady environments such as 5, 6 and 9 in this ranking.

Inspired by this study, further research can be done on the following topics: What are the anatomical and morphological differences between oleander grown in abundant shade or light, based on the difference in altitude? In fact, the leaves facing south samples only were taken from the localities, as the only factor, to select those who were more affected by light in this research. The other individuals which grew up in the shadows were ignored for this purpose.

Does leave structure change with bending in the wind? One might expect more wind at high elevations than that at low elevations. Also, the temperature at which leaves develop is probably lower at high elevations than that at lower ones. Another testable hypothesis is that higher UV (ultraviolet light) levels (at upper elevations) generate different leaf structure than that of those at sea-level.

Are the altitude-based changes also sourced from genetic (inherited) reasons or are they just examples of plasticity (non-inherited responses of plants to environmental conditions)? This question also emerges for future studies and could be addressed by common garden experiments, very easily done if *Nerium* can be propagated by cuttings. For this purpose, I suggest visiting the agricultural areas spread to different elevations such as in Mersin and Yalova cities, where it was grown as a park-garden plant for the detailed examination of the comparative anatomy of the Oleander parts such as leaves, flowers, pollen, etc.

CONCLUSION

Moreover, the reproductive biology of oleander plant can be investigated at different altitudes: Is there any differences in the production of flowers between Oleander shrubs at high elevations and near sea level? Are pollinators in high altitude areas different from those at low altitudes? Or, is it possible that the seasonality of fertilization is affected by the rise?

Consequently, this study has a distinct value for the first time that the changes in the anatomical and morphological structures of plant parts are expressed along the altitude gradient and also statistically compared and monitored. Therefore, I believe that this research will be a useful source of information for future research.

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