HISTOLOGICAL ANATOMY AND EPIDERMAL CHARACTERISTICS OF LEAVES IN SPECIES WITHIN THE FAMILY *NYMPHAEACEAE*

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Abstract

This is the first study about the micromorphology of aquatic plants of the family Nymphaeaceae in Albania. These species have the status of rare and endangered and have medicinal and decorative values. Given the current status and importance, the present paper provides important information about the taxonomy and philogeny of Albanian flora. The two species here studied have been collected from the "National Park of Lura", Albania. The histo-morphological techniques, i.e. the colloidal technique and leaf epidermis detachment technique were employed. Leaf morphology and anatomical characteristics can serve as bioindicators of plant response to altered environmental conditions and changing habitat use. The present study reports about the micromorphology of leaf and indicators of epidermis of leaf in *Nymphaea alba* L. and *Nuphar lutea* (L.) Sibth. & Sm. Parameters of stomatal density, stomatal index, stomatal size, trichomes density, trichomes index and druses density were measured. As a result, leaf epidermis indicators such as stomata, trichomes and druses (crystals) show variations within the same species but grown in two lakes with different microclimates thus displaying intraspecific diversity in these species. The presence of staurocytic stomata, stomatal clusters and druses density in both species is for the first time reported.
Key words: Aquatic flora, bioindicator, pollution, leaf, micromorphology

1. INTRODUCTION

The family Nymphaeaceae includes perennial aquatic plants with floating leaves. There are two species of this family: *Nymphaea alba* and *Nuphar lutea* in Albania (Xhulaj *et.al.*, 2013). Leaf morphology and anatomical features can serve as bioindicators of plant response to altered environmental conditions and changing habitat use, particularly air (Pal *et. al*., 2002;

Chaturvedi *et. al*., 2013) and soil (Abrams 1998; Tomasevic *et. al*., 2004; Sharma and Dietz, 2006). The morpho-anatomy of plant leaves can vary from contamination (Royer *et al*., 2008; Miller-Rushing *et al*., 2009; Pourkhabbaz *et al*., 2010). Differences in chemical composition and water depth, light intensity, and nutrient sediment conditions can lead to the genesis of specific morphotypes in plants that are not genetically determined and change rapidly with changing environmental conditions. Formal recognition of environmentally induced morphotypes has often resulted in an unwarranted evolutionary reversal and morphological sub-classifications of taxa (Kaplan 2002). In terms of *N. alba* species, its native population is declining rapidly in many European countries (Tomšovic 1988; Ejankowski and Małysz 2011). Despite the low number of European indigenous species, their high morphological polymorphism and plasticity have caused a persistent controversy regarding the divisions between taxa (Kabátová *et al*., 2014). As for *N. lutea* species, extreme morphological variability, uniform chromosome numbers and presumed hybridization have been obstacles to clearly understanding the relationships between taxa and to making a consistent taxonomic treatment (Padgett 2007). The epidermal parameters of the leaves, specifically the stoma and trichomes, their recognition and specification, serve the deeper characterization of a species and are an important indicator in determining the taxa in many plant families (Stuessy 2009). A change in stomatal density can be used as an indicator of environmental change (Case 2004). The presence or absence of trichomes and their density are influenced by various ecological factors, including tolerance to biotic and abiotic stresses (Bleeker *et. al*., 2012; Tian *et. al*., 2012). The specific mechanism that controls druses formation is unclear, but it has been suggested that a number of factors such as proteins, polysaccharides, and lipids or macromolecular membrane structures affect the shape and growth of crystals (druses). Druse may also have a purpose in regulating calcium (Horner and Wagner 1980; Arnott and Webb 1983; Webb, 1999). The different parameters of druse crystals are also related to the morphology of the leaves, as the density of these druse crystals is positively related to tissue density (Franceschi & Nakata, 2005). For these reasons, the micromorphological structure of the leaf and the stomata, trichomes and druses have been studied as an indicator of the relationship of *N. alba* and *N. lutea* species with the water ecosystem where they grow.

2. MATERIALS AND METHODS

Lura Lakes are a group of lakes of glacial origin, located in the eastern mountains of Lura and the Lura National Park in Albania. The aquatic plants studied here were collected in fresh condition, precisely from the “Liqeni i

Luleve" and the "Liqeni i Madh". The coordinates of these lakes determined by GPS are: "Liqeni i Luleve" N 41044`25 "E 20011`52.9" with an altitude of 1572 m above sea level and "Liqeni i Madh" N 41 ° 47'24.3 "E 20 ° 11'51.2" with an altitude of 1716 m above sea level.

Optic microscope, Olympus microscope connected to C200 camera, camera, lugol, blumethylene, transparent nail polish, fresh leaves, scotch tape, razor are in the present study used.

The colloidal technique. Five species for each genus were collected per habitat, and the morphological features of the leaf were analyzed. The leaf epidermis was cleaned up with water and then letting it dry with water. Once dried up, in the middle of the sheet, on the upper and lower epidermis, it was placed transparent nail polish. Once the nail polish is dried, it is placed on top of the painted part of the scotch glue, then the glue that takes the traces of the stomata and trichomes from the sheet is removed, and placed on the lame. (Http://www.zoo.utoronto.ca/able/volumes/copyright.htm). All the leaves underwent this process. The microscope first with a small zoom, and then with a 40x zoom was used.

The detachment technique. It consists of detachment of the leaf epidermis with a scalpel or razor and placing the removed part on the blade, where a lugol or blumethylene dye has been previously applied. In our case for the study of stomatal types in the leaves of species *N. lutea* the dyeing technique has been modified for a clearer appearance and for this reason the stomata have been dyed first with lugol and then with blumethylene. Clean areas are photographed with a camera and a computer-connected camera (Johnston and Watson 1976; Hilu and Randall 1984). The preparations are placed under a microscope for observation.

The transverse cutting technique was employed for the anatomical study of the leaves.

The transverse technique. The leaves were transversely incised to study of tissues and the construction of canned organs (leaf). These incisions are made with a sharp razor and with the help of the spinal cord when they become perpendicular to the axis of the organ to be cut. The material (leaf) is placed between the two pieces of the marrow of the appendage, which helps to make thin cuts.

The dyeing technique. The cuts are transferred to a clock glass with distilled water, and then passed to a clock glass with safranin where they are left for 2-3 minutes to be colored. The same procedure is followed for dyeing with toluidine. After dyeing, the cuttings are sent back to a watch glass with clean water to be rinsed. After this action, the cuts are ready to be observed.

The preparation and observation. In the center of the blade we release a drop of glycerin, place the thinnest incisions and cover them with lamellae (Topuzi 2005). The Olympus microscope with a 40x lens was involved for the observation of the preparations. The Olympus camera was used to photography the preparation. The photos were edited through the Microgliciel and Photoshop software.

3. RESULTS

The figure 1 depict the "Liqeni i Luleve" and "Liqeni i Madh". The area around the "Liqeni i Luleve" is characterized by deforestation. Light intensity is high, erosion occurs and pollution could be noted. Erosion *a*nd *sediment* transport into the lake is the source of water depth reduction and eutrophication "Liqeni i Madh" does not suffer from erosion. Here the water is clearer and the water depth is greater and light intensity lower because of forestation.

Fig. 1: a. "Liqeni i Madh"; **b.** "Liqeni i Luleve".

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Regarding the micromorphological structure of the leaf of the species *N. alba*, the upper epidermis covered by the thin cuticle could clearly distinguished. The transverse anatomy of the leaf appears to have a polygonal spongy tissue structure composed of typical aerenchyma, and the upper mesophyll was occupied by homogeneous palisade parenchyma. Beneath the upper epidermis, there is the palisade mesophyll which occupies about 1/3 of the leaf, and below it is the spongy mesophyll which occupies most of the leaf, about 2/3 of it.

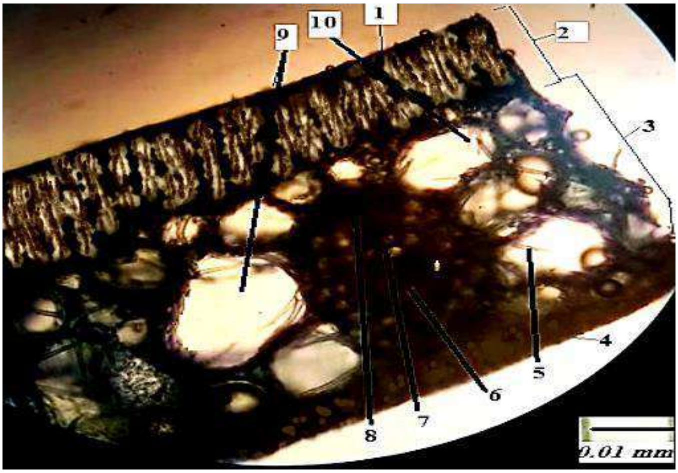


Fig.2: Cross section of leaves in species *Nymphaea alba*; **1.** upper epidermis; **2.** palisad mesophyll; **3.** spongy mesophyll; **4.** lower epidermis. **5.** elonged sclereide; **6.** phloem; **7.** Xylem, **8.** phloem (**6-8** - forms bicolateral vascular bundles); **9.** Air space; **10.** Asterosclereides (star-shaped trichosclereides).

Aerenchymes with different and relatively large dimensions could be distinguished in spongy mesophyll. In these air spaces, sclereides could also be distinguished. They are extensions of spongy mesophilic cells and their tops extend to the air spaces. The sclereides found on these leaves are of the elongated sclereides type and star-shaped sclereides with branching otherwise known as asterosclereides. Vascular bundles of the bicolateral type extend along the width of the spongy mesophyll. The last layer of the leaf is the lower epidermis, which is usually submerged in water (Fig. 2). The leaves of *N. alba* are of the epistomatic type, as they have stomata only in the upper epidermis. Anomocytic (white arrow), actinocytic (green arrow) and staurocytic (blue arrow) are types of stomata. Trichomes (black arrow) and druse (red arrow) are found in the lower epidermis of the leaf. The presence of large stomatal clusters in this species has also been encountered. Stomatal clusters are contiguous (red circle) and non-contiguous (black circle), where non-contiguous clusters dominate (Fig. 3). Based on statistical processing, both populations of *Nymphaea alba* show variability of stomatal parameters. The leaves of *N. alba* are of the epistomatic type as they have stomata only in the upper epidermis.

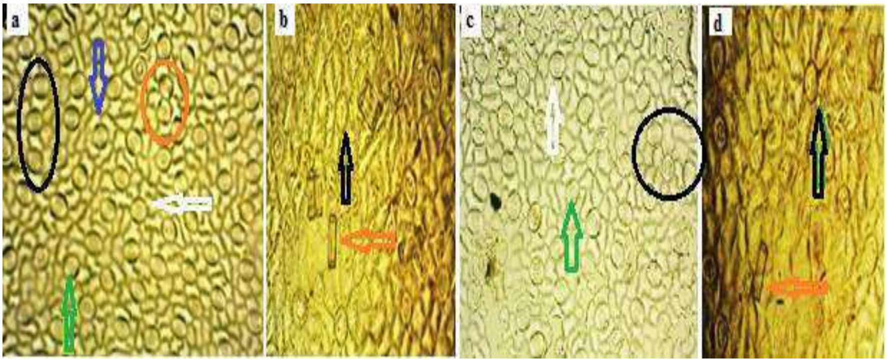
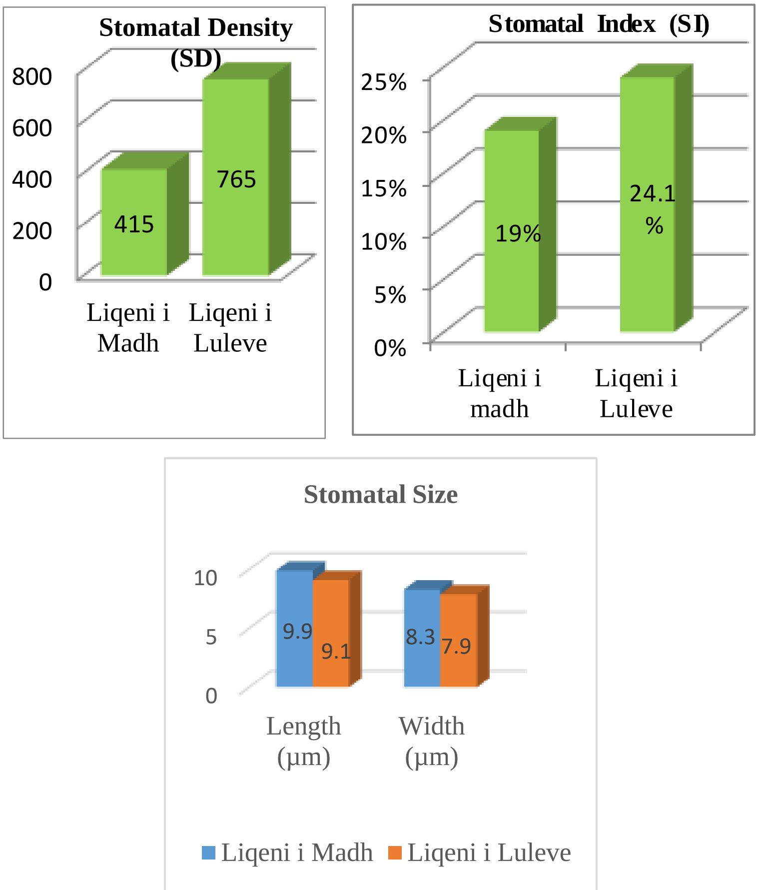


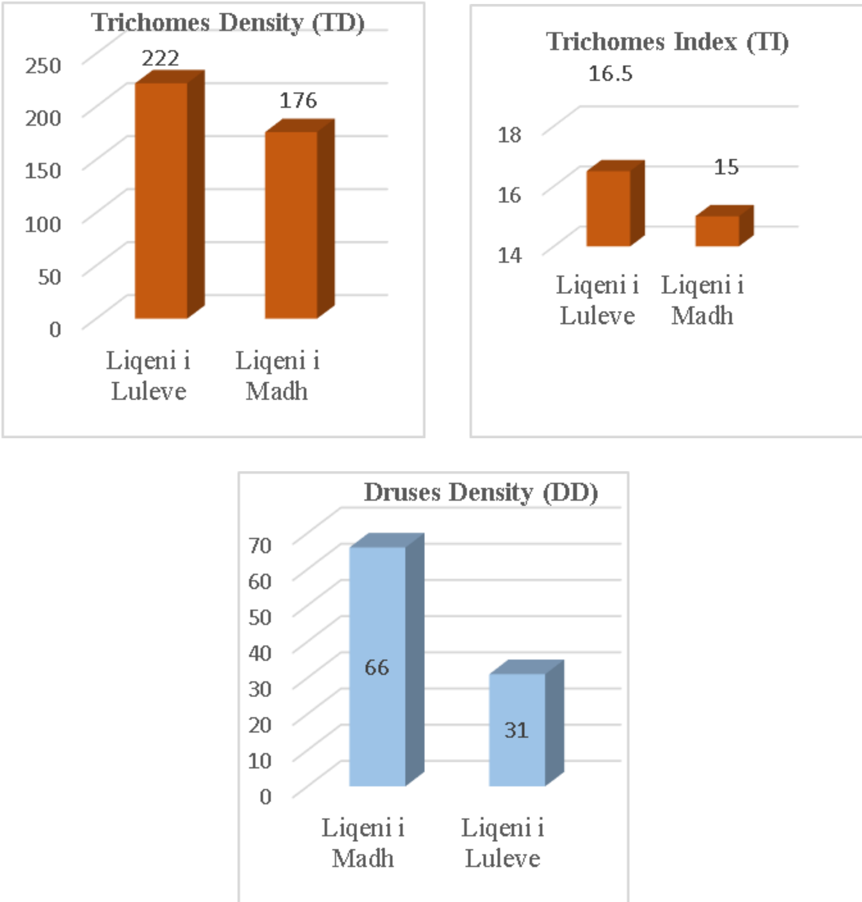
Fig. 3. Stomata, trichomes and druses in both population of *N. alba*. **a.** stomata of the upper epidermis in the "Liqeni i Luleve", **b.** trichomes and druses on the lower epidermis in "Liqeni i Luleve", **c.** stomata in the upper epidermis in the "Liqeni i Madh", **d.** trichomes and druses on the lower epidermis in the "Liqeni i Madh".

The mean value of Stomatal Density (SD) for the "Liqeni i Madh" population is 415 stomata/mm^2. The mean SD value for the "Liqeni i Luleve" population is 765 stomata/ mm^2. In addition to the variation between the two populations in terms of SD, the variation regarding the Stomatal Index (SI) parameter is also reported. The mean SI value for the "Liqeni i Madh" population is 19%. The mean SI value for the "Liqeni i Luleve" population is 24.1%. Regarding the stomatal size, the variation between the two populations appears both in the length of the stomata and in their width. The species of "Liqeni i Madh" appear with stomata of greater length and width than the species of Liqeni i Luleve. The mean value of stomata length for the population of the "Liqeni i Luleve" is smaller (9.1 µm) than the mean value of the length of stomata for the population of the "Liqeni i Madh" (9.9 µm). The mean value of stomata width for the population of Liqeni i Luleve is smaller (7.9 µm) than the mean value of stomata width for the population of the "Liqeni i Madh" (8.3 µm) (Graph 1).



Graph 1. Mean value of stomatal density, stomatal index and stomatal size in both population of *N. alba.*

The peltate glandular trichomes could be found. Statistical data about the populations of *Nymphaea alba* show variability of trichome parameters which could be noted in the figure 3, where the distribution of trichomes in the lower epidermis occurs.



Graph 2. Mean values of trichomes and druses. **a.** density of trichomes; **b.** index of trichomes; **c.** density of druse in both population of *Nymphaea alba* L.

The Trichomes Density (TD) represents the number of trichomes per 1 mm^2. The mean TD value for the “Liqeni i Madh” population is lower (176 trichomes/mm^2) than the mean TD value for the “Liqeni i Luleve” (222 trichomes/mm^2). The Trichomes Index (TI), which represents the percentage occupied by trichomes per 1 mm^2 of leaf area, was also measured. Regarding the species of the “Liqeni i Luleve”, TI is higher. The mean TI value for the “Liqeni i Madh” population is lower (15%) than the mean TI value for the Liqeni i Luleve population is higher (16.5%). In terms of druses, Druses Density (DD) represents the number of druses per 1 mm^2. The mean DD value for the “Liqeni i Luleve” population is lower (31 druses/mm^2) than the mean DD value for the "Liqeni i Madh" population is higher (66 druses/mm^2) (Graph 2).

The Figure 4 depicts the micromorphological structure of the leaf in species of *Nuphar lutea*. The upper epidermis is covered by the cuticle, the palisade mesophyll and the spongy mesophyll and the lower epidermis could be clearly distinguished. In spongy mesophyll, elongated air spaces (aerenchyma) could be distinguished. Vascular bundles extending along the width of the spongy mesophyll could be found. They are of the collateral type where the xylem stands opposite the phloem. The last layer of the leaf is the lower epidermis, which has 2 - 3 cell layers. The lower epidermis is usually submerged in water. The leaves of *N. lutea* are large and heterophilous, of the epistomatic type, i.e. the stomata are only in the upper epidermis.

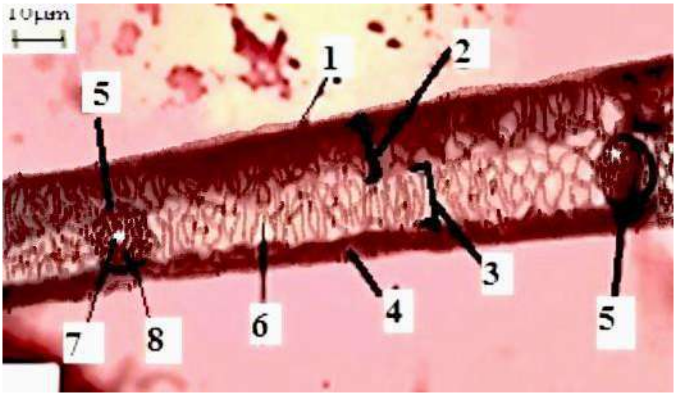


Fig. 4: Cross section of the leaf of the species *N. lutea*. **1.** Upper epidermis; **2.** Palisade mesophyll; **3**. Spongy mesophilia; **4.** Lower epidermis; **5.** Vascular bundle (surrounded); **6**. Aerenchyma; **7**. Xylem; **8.** Phloem; (zoom 10x).

The stomata encountered in these plants are of the anomocytic (Fig. 5 b) and staurocytic type (Fig. 5c). The presence of staurocytic spores in the leaf of *N. lutea* has not been previously mentioned by the scholars studying the family Nymphaeaceae. The Figure 5d depicts the trichomes (black arrow) and druses (red arrow) in the lower epidermis of the leaf. The presence of large stomatal clusters in this species has also been encountered. Stomatal clusters are contiguous (red circle) and non-contiguous (black circle), but non-contiguous clusters prevail (Fig. 5a). Based on the data obtained from the statistical processing, both populations of *Nuphar lutea* present variability of stomatal parameters.

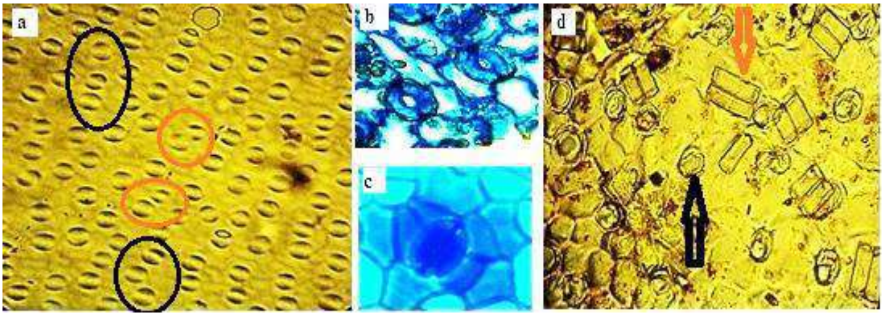
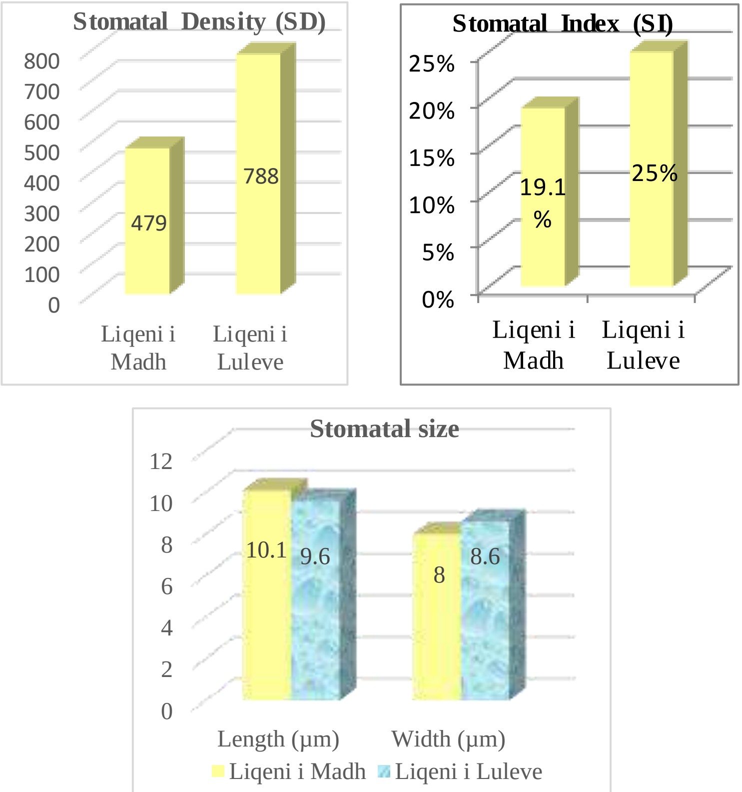


Fig. 5: View of the upper epidermis and lower epidermis of leaves in species *N. lutea*. **a.** stomata in the upper epidermis; **b.** anomocytic stomata; **c.** staurocytic stoma; **d.** trichomes and druse in the lower epidermis.

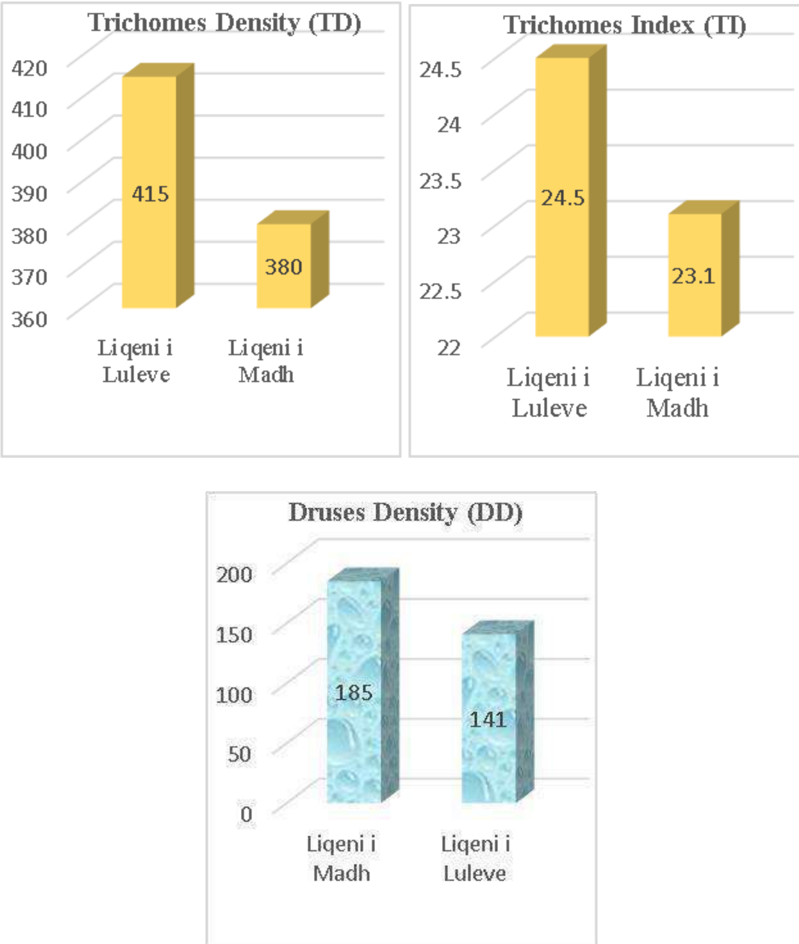
In addition to the variation between the two populations in terms of DS, the variation regarding the Stomatal Index (SI) was also reported. The mean SI value for the "Liqeni i Madh" population is lower (19.1%) than the mean SI value for the Liqeni i Luleve population (25%). In terms of stomata size, the variation between the two populations is manifested in both the length and width of stomata (Graph 3).

The mean value of stomata length for the population of the "Liqeni i Luleve" is 9.6 µm. The mean value of stomata length for the population of the "Liqeni i Madh" is 10.1 µm. The mean value of stomata width for the population of "Liqeni i Luleve" is higher (8.6 µm) and the mean value of stomata width for the population of the "Liqeni i Madh" is smaller (8.04 µm). Regarding trichomes, from the analysis of data as a result of statistical processing the two populations of *Nuphar lutea* present variability of trichomes parameters. The mean TD value for the "Liqeni i Madh" population is lower (380 trichomes /mm^2) and the mean TD value for the Flower Lake population is higher (415 trichomes/mm^2) (Graph 3).



Graph 3. Mean values of dental density, dental index and stomata size in both populations of *N. lutea.*

The mean TI value for the "Liqeni i Madh" population is lower (15%) than the mean TI value for the Liqeni i Luleve population (16.5%). In terms of druses, they represent variability in the number of druses in each population. A parameter measured in relation to drues is the density of druses (DD) which represents the number of druses per 1 mm^2. This parameter is presented for the first time. The mean DD value for the "Liqeni i Luleve" population is lower (141 druses/mm^2) than the mean DD value for the "Liqeni i Madh" population (185 druses/mm^2) (Graph 4).



Graph 4. Mean values of trichomes density, trichomes index and druses density in both populations of *N. lutea.*

4. DISCUSSIONS

The upper epidermis of the *Nymphaea alba* leaf is covered by a thin cuticle. Also the polygonal structure of the spongy tissue composed of typical aerenchyma, and the upper part of the mesophyll occupied by homogeneous hepatic parenchyma are also described by Gonzalez (2002) for the order Nymphaeales. The sclereides found on these leaves are of the elongated type and star-shaped sclereides with branching otherwise known as asterosclereides. Asterosclereides in aerenchymes have also been reported by

Conard (1905) and Sculthorpe (1967). The vascular bundles are of the bicolateral type, which have also been reported by Kaul (1976). In the family Nymphaeaceae, anomocytic stomata (Solereder 1908; Metcalfe and Chalk 1950; Carpenter 2006) and actinocytic stomata (Carpenter 2006) have been reported. While the presence of staurocytic stomata is reported for the first time in the family Nymphaeaceae and in the genus Nymphaea. The presence of contiguous and non-contiguous stomatal clusters has also been encountered. Both populations of *Nymphaea alba* present variability of epidermal indicators in leaves. These changes consist of marked variations in terms of stomatal density, stomatal index, stomatal size, trichomes density, trichomes index, and druses density. This shows marked intraspecific diversity between the population of *N. alba* in the "Liqeni i Luleve" and the population of *N. alba* in the "Liqeni i Madh". Regarding the density of trichomes Carpenter (2006) reported that the density of trichomes in the genus Nymphaea is on average about 188 trichomes/mm^2 while in our study the density of trichomes in the population of *N. alba* in the "Liqeni i Luleve" is on average 222 trichomes / mm^2 and in the "Liqeni i Madh" is 176 trichomes/mm^2.

The micromorphological structure of the leaf of *Nuphar lutea*, in the spongy mesophyll has air spaces (aerenchymes) with elongated shapes. Coan *et. al*., (2002) said that the presence of many separate aerenchymes as chambers presents an important adaptation because it facilitates the storage of a minimum amount of oxygen needed in aquatic environments and enables the transport of oxygen from the leaves to the roots. Collateral vascular bundles are distinguished in spongy mesophyll. For Sculthorpe (1967), a sufficient number of lacunar protoxylames observed in conductive herds may represent the only structure that transports water to many aquatic species.

The presence of staurocytic stomata is reported for the first time in even in the genus Nuphar. The presence of contiguous and non-contiguous stomatal clusters has also been encountered. Both populations of *Nuphar lutea* present variability of epidermal leaf indicators. These changes consist of marked variations in terms of stomatal density, stomatal index, stomatal size, trichomes density, trichomes index, and druses density. This shows marked intraspecific diversity between the population of *N. alba* in the "Liqeni i Luleve" and the population of *N. alba* in the "Liqeni i Madh".

Based on Monteiro (2001) and Freire *et al*., (2007), these histological structures have adapted to changing environmental conditions, which made them reflect the changes in their morphology and structure. Case (2004) said that a change in stomatal density can be used as an indicator of environmental change. Some researchers have shown that stomatal density varies in response to water availability (Edward and Meidner 1978), light intensity (Retallk, 2001; Lu *et al*., 1993), temperature (Ciha and Brun, 1975), geographical location (Retallk, 2001) and CO_2 concentration (Bristow and Looi 1968; Woodward 1987; Woodward and Bazzaz 1988).

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