STRUCTURE AND MICROMORPHOLOGY OF THE LEAF IN SOME TAXUS SPECIES

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Abstract. In this paper leaf structure of 4 Taxus species and varieties (*Taxus baccata, T. baccata* var. *fastigiata, T. brevifolia, Taxus cuspidata*) was investigated. Histological and micromorphological studies were carried out by light and scanning electron microscopy. From the histo-anatomical point of view, the leaf shows a general structural pattern with palisade and spongy parenchyma. This is common to all investigated taxa with quantitative variations. The stomata are located only in the lower epidermis (hypostomatic leaves), being disposed on 2 bands, with different widths, on one side and the other of the midrib. The papillae of the cells from the lower epidermis are also visible next to the median rib, with the exception of the species *T. brevifolia*. The palisade parenchyma is bilayered, with short cells. The structural characters of the leaves have taxonomical significance.The importance of the structural features of the leaf in the adaptation of the species to the living environment were emphasized.

Key words: anatomy; epidermis; papillae; palisade parenchyma; micromorpgology; leaf; Taxus.

INTRODUCTION

The Taxaceae family, commonly called the yew family, includes six genera and about 30 species. They are branched small trees and shrubs with evergreen leaves, often twisted to appear 2-ranked [18]. The genus Taxus is a small group of gymnosperms representing less than 2% of gymnosperm species; it is widely cultivated as an ornamental shrub, in the northern hemisphere [15].

In the same time, Taxus species have important medicinal uses; Paclitaxel (Taxol®) extracted from *Taxus brevifolia* has proven to be useful in the treatment of cancer (ovarian, lung, esophagus, etc.), acting on cells in metaphase, by blocking the normal dynamics of the division spindle [1]; this valuable compound was also extracted from the young stems and leaves of *Taxus cuspidata* [22]. It should be mentioned that they can contain strong toxins: *Taxus baccata* contains the toxin taxine B. Between 42 and 91 g of yew leaf is lethal to a 70-kg adult [16]. Therefore, the administration of preparations obtained from plant material from these species must be done with caution, under the guidance of specialists.

Taxus baccata is a tertiary relict, with the status of a threatened plant, included in conservation programs in many European countries [12]. *Taxus baccata* var. *fastigiata* is cultivated variety of *T. baccata,* with column-shaped growing, which remains narrow during their lifetime. *T. brevifolia* is native from the western part of the USA and Canada, and *T. cuspidata* originates from Asia - Japan, Northern China and Eastern Russia [10].

The histo-anatomical and micromorphological investigation upon the Taxaceae family was carried out by some authors [2, 5, 17, 19, 23]. General data could be finding in some clasical botanical traits. In 1996, Strobel and Hess [20] have performed a scanning electron microscopy study of Taxus leaves related to taxonomy and revealed features that appear useful in the clasification of this tree species, which yields an important anticancer drug, taxol. In 1997, Di Sapio *et al.* [2] have made some anatomical investigations analysis upon *Taxus baccata* bark and leaves (the study of leaf epidermis, stomata, leaf architecture and internal structure). Ghimire *et al.* [5] highlighted the importance of anatomical characters in the research of phylogenetic relationships in Taxaceae.

The purpose of this paper is to present a detailed description of the anatomy, micromorphology and biometry of the leaf from the Taxus species cultivated in the Bucharest Botanical Garden. Structural adaptations to the living environment were emphasized; also, the obtained data can serve as support for the taxonomic and phylogenetic analyzes referring to this genus.

MATERIALS AND METHODS

Plant material: The plant material was represented by mature leaves from 4 species and varieties of Taxus genus: *T. baccata, T. baccata var fastigiata, T. brevifolia* and *T cuspidata*, harvested from the Botanical Garden "D. Brandza" Bucharest. The biological material was preserved in 70% ethyl alcohol.

Histo-anatomical analysis: free hand sections were performed using a razor blade. The sections were coloured with Iodine Green and Ruthenium Red. Photographs were taken with an Olympus E-330 photo camera, using an Olympus BX41 research microscope, in normal and polarised ligth.

Scanning electron microscopy (SEM) investigations: Free air-dried leaf samples were examined by scanning electron microscope (Tescan Vega II SBH) (from the Electron Microscopy Laboratory, Faculty of Biology, Alexandru Ioan Cuza University of Iași).

Microorphological and anatomical traits measurements:

In this paper a total of 8 traits related to anatomy and micromorphology of leaves were selected for investigation.

The measurements were made using the IC Measure - The Imaging Source software.

Histo-anatomical measurements: Pallisade parenchyma cells layers (number), palisade parenchyma cells height, upper and lower epidermis cells high were investigated; for each parameter, 20 measurements were made (n =20).

Micromorphological measurements: The dimensions of the stomata were measured (the length being considered the size of the stomata, including the subsidiary cells, in a plane parallel with the median rib, and the width the size of the stomata in a plane perpendicular to the median rib); for each parameter, 20 measurements were made (n =20). The number of subsidiary cells was determined by averaging cells from 10 stomata/sample. The density of stomata was determined by measuring 10 different fields/species (only the areas of the lower epidermis where the rows of stomata are located were analyzed). The width of the bands with stomata was measured in 10 different points, both on the right and on the left of the median rib.

Based on the obtained morphometric data, PAST, a freeware data analyzer was used to create a dendrogram that reflects the distance between the analyzed species. For data calculation in the hierarchical clustering analysis, the Euclidean similarity index and the bootstrap values (Boot N - set to 1000) were used.

RESULTS

Histo-anatomical results:

The leaves are linear to lanceolate, and have pale green or white stomatal bands on the undersides. In the analyzed species, the leaves showed a typical bifacial dorsiventral heterofacial structure (Fig. 1, 2).

The upper epidermis is made up of a single layer, with approximately isodiametric cells, slightly elongated tangentially. They have thickened external walls, covered by a thick cuticle, without striations. At the level of the upper epidermis no stomata are observed.

The lower epidermis is also single-layered, consisting of isodiametric cells that present prominent papillae, in the middle part of the leaf and in the lateral parts. The cuticle is thick in all investigated species. The presence of papillae at the level of the external walls of the cells in the lower epidermis is not uniform in the analyzed species: thus in *T. baccata* the papillae are observed both in the central part (next to the median rib) and in the lateral parts (up to the last 7-9 rows of cells, located towards the edge of the sheet, where they are no longer visible). In *T. baccata* var.

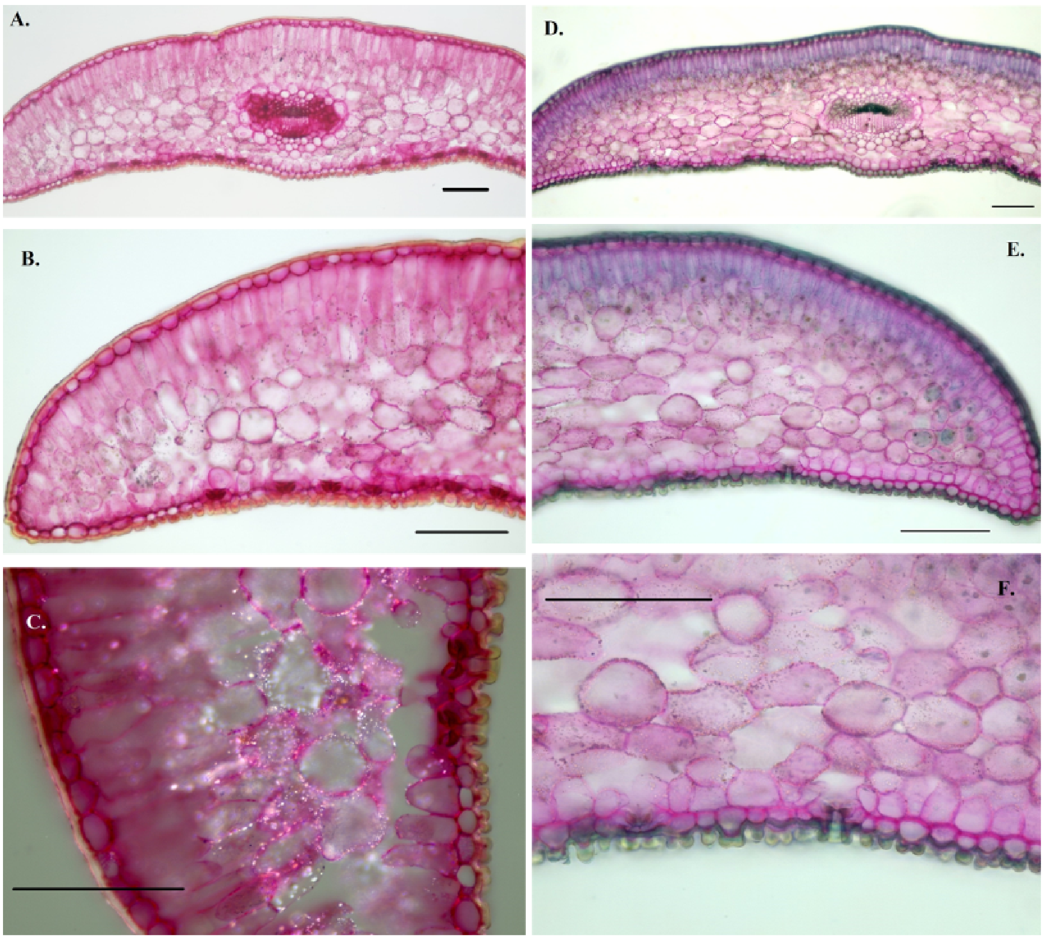

Figure 1. Cross sections through leaves: A-C *Taxus baccata,* D-F *T. baccata* var *fastigiata* (C – photo taken in polarized light). Scale bar =100 µm.

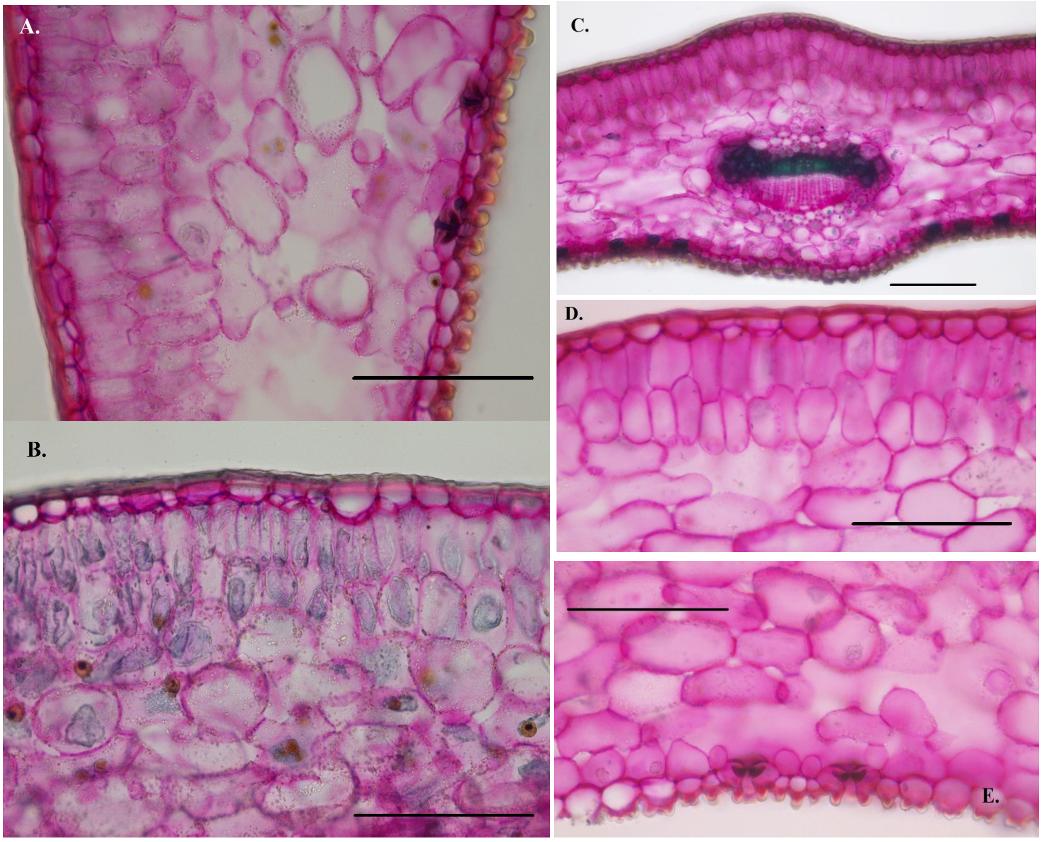

Figure 2. Cross sections through leaves: A-B *Taxus brevifolia,* C-E *T. cuspidata.* Scale bar = 100 µm.

fastigiata, the papillae have a similar distribution, but they extend almost to the edges of the lamina - only on the last 2-3 rows of cells from the edge being less noticeable). In *T. brevifolia*, in the area of the midrib, the papillae are strongly flattened and missing on some cells. In *T. cuspidata*, the papillae are found along the entire length of the lower epidermis, with the exception of the last 4-5 rows of cells in the margin where they suddenly disappear.

The stomata are present at the level of the lower epidermis and are arranged in rows, in the form of two wide bands, on one side and the other of the median rib. In cross-section, the stomatal apparatus appears composed of 2 guard cells with strongly thickened dorsal and ventral walls, located completely below the level of the epidermis. Subsidiary cells present obvious papillae that delimit a narrow suprastomatal chamber.

In all analyzed species the hypodermis is missing.

The mesophyll is differentiated into palisade parenchyma located under the upper epidermis and spongy parenchima under the lower one.

The palisadic parenchyma is bi- or unilayered (the structure of the palisadic tissue is shown in Table 1); its cells are cylindrical, perpendicular to the upper epidermis, longer in *T. baccata* and shorter in *T. brevifolia*. The spongy parenchyma is multi-layered, consisting of 7-9 layers of cells in *T. baccata* var *fastigiata* or 4-6 layers in *T. baccata, T. brevifolia* and *T. cuspidata*. The cells are large, almost isodiametric or elongated, with moderate air spaces between them.

Observations of the sections under polarized light revealed, in all investigated species, numerous small calcium oxalate crystals, especially at the level of the spongy parenchyma (small amounts are also observed in the palisade parenchyma). The localization of these crystals is both intra and especially extra cellular

Table 1. Summary of leaf anatomical features of investigated Taxaceae species

Taxa	Anatomical feature				
	Epidermis cell wals	**Pallisade parenchyma cells layers**	**Pallisade parenchima height (µm)**	**Upper epidermis cells high (µm)**	**Lower epidermis cells high (µm)**
Taxus baccata	Papillate (lower epidermis), moderate thick (upper epidermis)	2	47.349 (1SD: 9.06)	19.69 (1SD: 1.70)	18.286 (1SD: 2.65)
Taxus baccata var. *fastigiata*	Papillate (lower epidermis), thick (upper epidermis)	1 (the second one is discontinuous)	35.915 (1SD: 7.35)	18.42 (1SD: 1.67)	18.053 (1SD: 1.15)
Taxus brevifolia	Papillate (lower epidermis), moderate thick (upper epidermis)	2	34.367 (1SD: 3.84)	17.439 (1SD: 2.00)	21.013 (1SD: 1.37)
Taxus cuspidata	Papillate (lower epidermis), moderate thick (upper epidermis)	2	36.231 (1SD: 5.59)	18.556 (1SD: 1.30)	19.01 (1SD: 2.57)

Note:Values are presented as means ± SD

(embedded in the outer face of the cell walls of the spongy parenchyma).

The conducting tissues are arranged in the form of a simple central bundle, of the closed collateral type. The xylem is formed by tracheids, with a reduced diameter, arranged in rows. The phloem consists of perforated cells and rows of parenchymal cells. A typical endoderm is missing; large round parenchymal cells are present, which partially surround the conducting fascicles and which can be interpreted as an endodermoid. The transfusion parenchyma is located laterally, being made up of cells with cellulosic walls in *T. baccata*, slightly lignified in *T. baccata* var *fastigiata* and *T. brevifolia* or more intensely lignified in *T. cuspidata*. At the periphery of the vascular bundles, bands of obliterated and closed protophloem cells could be observed. The resin canals are missing.

Some morphometric data regarding the anatomical characteristics of the leaf in the investigated taxa can be seen in Table 1.

Micromorphological results:

Scanning electron microscope (SEM) investigations focused on the areas of the leaf where the stomata are located (the two bands on the lower epidermis) (Fig. 3, 4). In these regions, the stomata are arranged in rows - approximately parallel, on one side and the other of the median rib. The stomata are located below the level of the epidermis, the guard cells being surrounded by a variable number of subsidiary cells (4-6, depending on the species - see the data in Table 2). We observed the highest number of subsidiary cells in *T. cuspidata*, and the lowest in *T. brevifolia* (*T. baccata* and *T. baccata* var. *fastigiata* registering intermediate values).

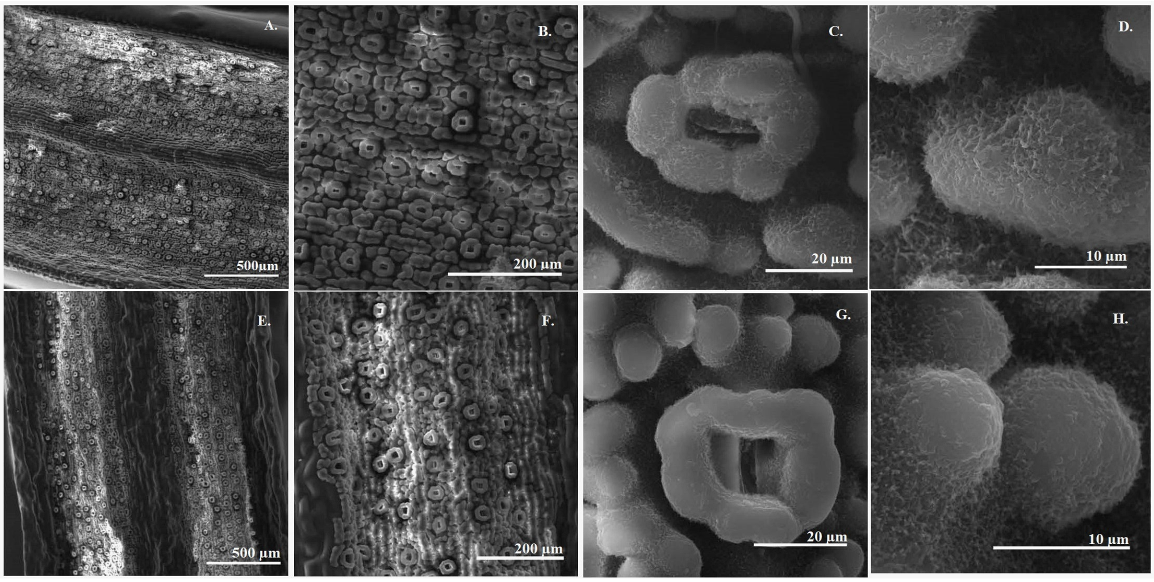

Figure 3. Leaf micromorphological aspects of the lower epidermis: A-D *Taxus baccata,* E-H *T. baccata* var *fastigiata* (A, E – general aspect, B, F – stomatal bands, C, G – stomatal apparatus, D, H – epidermic cell papillae)

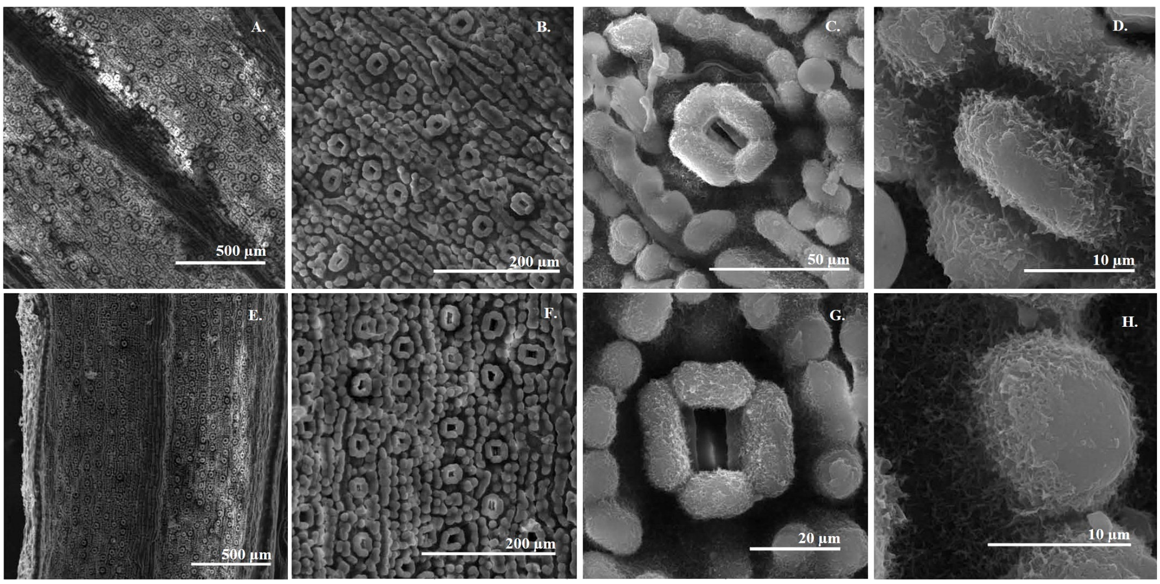

Figure 4. Leaf micromorphological aspects of the lower epidermis: A-D *Taxus brevifolia,* E-H *T. cuspidata* (A, E – general aspect, B, F – stomatal bands, C, G – stomatal apparatus, D, H – epidermic cell papillae)

Table 2. Summary of leaf micromorphological features of investigated Taxaceae species

Taxa	Micromorphological feature				
	Somata length (µm)	**Stomata width (µm)**	**Subsidiary cell number/stomata**	**Stomata density (number/mm²)**	**Stomatal band width (µm)**
Taxus baccata	34.85(1SD:2.28)	27.2(1SD:3.34)	4.58(1SD:0.51)	175.38 (1SD: 22.58)	563.923 (1SD:25.38)
Taxus baccata var. *fastigiata*	35.92(1SD:5,19)	38.34(1SD:3.53)	4.66(1SD:0.49)	180.55 (1SD: 28.52)	488,677 (1SD:21.14)
Taxus brevifolia	32.67(1SD:3.26)	35.23(1SD:2.51)	4.16(1SD:0.38)	134.14 (1SD: 26.49)	732.656 (1SD:29.15)
Taxus cuspidata	31.32(1SD:3.81)	33.2(1SD:3.45)	5.33(1SD:0.65)	160.36 (1SD: 25.72)	478.138 (1SD:39.57)

Note: Values are presented as means ± SD

The micromorphological analyzes were performed on material dried directly in air, with the aim of avoiding the contact of the epicuticular wax with organic solvents that could change its relief (the presence of thick cell walls in the epidermal cells facilitates this type of analysis, without inducing significant artifacts in the foliar micromorphology).

In *T. baccata* and *T. baccata* var. *fastigiata*, the epicuticular wax observed on the papillae of epidermal cells and subsidiary cells presented a slightly amorphous appearance; in contrast, the epicuticular wax observed on the same structures in T. brevifolia and T. cuspidata has a much more ordered structure, in the form of anastomosed tubules (in *T. brevifolia* they are visible on the entire surface of the epidermal cells, with a visible flattening at the tip of the papillae, generated by direct interaction with the external environment - justifiable considering that the analyzed leaves were mature leaves).

Some morphometric data regarding the micromorphological characters of the leaf in the investigated taxa can be seen in Table 2.

The numerical data obtained in the anatomical and micromorphological analyzes were used to build a dendrogram to illustrate the degree of similarity between the Taxus taxa analyzed (Fig. 5). According to the cluster analysis, *Taxus baccata* and *T. baccata* var *fastigiata* are the closest to each other (an expected fact, because it is a species and its variety), and these two are close to *T. cuspidata*, with which they form a common cluster; *T. brevifolia* is much more distant from the other 3 taxa. This is demonstrated by the dendrogram produced by cluster analysis of the 8 anatomical and micromorphological characters investigated.

DISCUSSION

The leaves from the Taxus species analyzed presented a common structural plan, the differences observed between them being especially quantitative. Al the analized taxa were colected from Botanical Garden of Bucharest University, because if possible, it is better to compare plants grown under identical conditions to test the value of anatomical features [6]. Like most species of gymnosperms with evergreen leaves [5], the taxa analyzed in this paper also have visible xerophytic traits. Although they come from very distant geographical areas, the analyzed species show a high homogeneity of anatomical and micromorphological characters, denoting adaptations to somewhat similar living environments.

The reduced dimensions of the leaves (especially the width - which brings the cells of the mesophyll to a small distance from the conducting testes) represent a strategy of water conservation in the body of the plant [21, 25]. Taking into account the geographical origin of the analyzed species, in temperate regions and relatively high mountain areas, with cold periods in the year, the morpho-anatomical adaptations reflect the existence of a physiological drought, to which the plants respond with strategies to reduce water losses. Taxus species are known to be resistant to shading [23], and for this reason the characters of xerophytism must be in balance with those typical of shade resistance [3]. The external walls of the epidermal cells are thick, covered with thick cuticle, but this is balanced by a usually bilayered or unilayered palisade tissue, with rather short cells (36-47 µm).

The stomata are deeply sunken in the mesophyll in all investigated taxa. However, there are differences

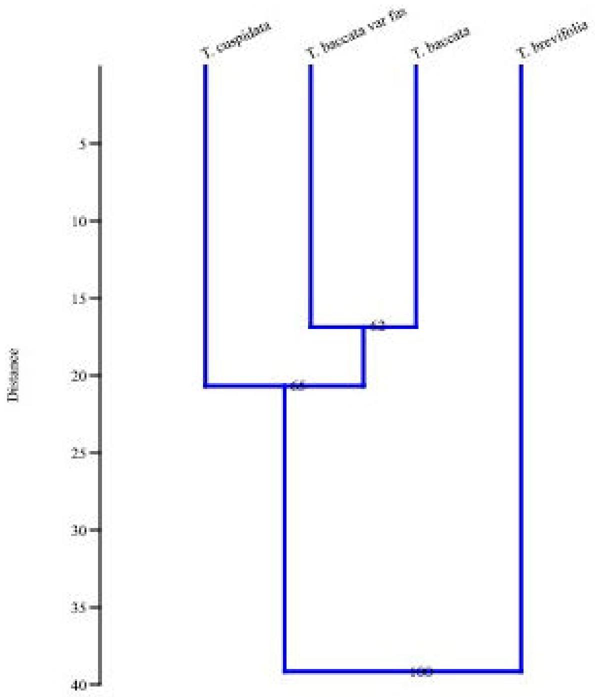

Figure 5. Hierarchical clustering dendrogram of examined taxa based on the mean value of 8 anatomical and micromorphological traits (Euclidean Boot N 1000)

between the sizes and density of stomata in these species: for example, the largest stomata and their highest density/mmp were observed in *T. baccata* var *fastigiata* - a cultivated variety, for which adaptation to water retention is less important than in wild species.

No papillae were observed in the cells of the upper epidermis in any of the analyzed taxa, although they were reported by Elpe [4] in *T. brevifolia*. These differences can be induced by populational variations because the material was collected, in both situations, from the European area.

In the analyzed species, we found the absence of foliar sclereids, which are described in other taxaceae genera; also, the endoderm is not a typical one, we can observe at most an endodermoid, formed by parenchyma cells of larger sizes, arranged in a relatively orderly manner. Elpe [4] found the lack of endoderm in species of the Taxus genus - in fact, the lack of cells with secondary chemically modified walls, because they carried out investigations under the fluorescence microscope. The lack of an apoplastic barrier in the way of water loss through transpiration can be correlated with a lower resistance of taxaceae to drought, compared to other gymnosperm species, with more pronounced xerophytic features [11].

Diffusion parenchyma is present only in the lateral parts of the conducting bundles, with a different degree of lignification in the investigated species; the central cylinder is not as well highlighted as in other gymnosperms, where it represents a characteristic of the leaf anatomy [3]. The distribution of stomata in two bands located near the mid rib was associated with the presence of the transfusion parenchyma in a lateral position, related to the conducting bundle in the leaf [13].

The role of the transfusion parenchyma is documented as a barrier to the crushing of wood vessels, if the water loss occurs before the stomata closure takes place [24]; these buffer the water potential of the xylem and protect the xylem from increasing tensions. The stomatal closing process has a lower reaction speed in gymnosperms compared to angiosperms, due to the presence of the suprastomatal chamber (which limits its interaction with the external environment) [9]. The weaker development of the diffusion parenchyma in Taxus species (especially in *T baccata* and *T. baccata* var. f*astigiata*) suggests that it may offer less protection to the xylem [24].

The presence of calcium oxalate crystals in gymnosperms (and not only) is associated with defense mechanisms - in this case it is possible that their presence represents a means of defense against herbivorous animals, especially in Taxus species that lack resiniferous ducts. Hudgins *et al.* [7] attributed a similar defense function to the crystals in the bark of some species of Pinaceae, which would have a protective role against bark-boring insects.

Regarding the taxonomic interrelationships between species of the Taxus genus, they have been analyzed by several authors, especially using genetic data, the chloroplast genome, simple [8], or in combination with nuclear markers [14], or nuclear ribosomal DNA [10]. Most of the authors who performed phylogenetic analyzes considered that there are few morphological or structural characters on the basis of which Taxaceae species can be separated. Elpe et all. [4] carried out phylogeny investigations on some species from various genera of Taxaceae, mainly using anatomical characters observed under the fluorescence microscope.

The dendrogram constructed in this paper, based on anatomical and micromorphological characters, shows a similar arrangement of the species *T. baccata, T. brevifolia* and *T. cuspidata* (more precisely - a greater proximity between *T. baccata* and *T. cuspidata*, which are distant from *T . brevifolia*), like those obtained by the authors mentioned above. This demonstrates the usefulness of structural characters in the analysis of phylogeny in gimonsperms (and not only).

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