Ontogenetic transformation in the anatomical structure of leaf-like formations in some herbaceous Boraginaceae growing under various edaphoclimatic conditions

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Summary: A comparative anatomical analysis of assimilating leaves, including cotyledons, in nine herbaceous Boraginaceae was undertaken. In their microstructural organization, a number of significant heterogeneous traits, connected with their habitats, environment, life-forms, ontogenetic and morphogenetic stages were revealed. The adaptation strategy for every species was identified. Three ecological groups are clearly defined: mesophytes, mesoxerophytes and hygromesophytes. In some species, the revealed age heterogeneity in consequently emerging leaves appears as early as at the seedling stage, whereas in other ones the ontogenetic transformations run smoothly, i.e. heteroblastic and homoblastic types of development are evident. Differences in the anatomy of leaf-like formations in the studied Boraginaceae species revealed in the course of the present study display the possibility of taxonomic identification, starting from the earliest ontogenetic stages.

Keywords: Boraginaceae, ecology, life-forms, anatomy, ontogenetic transformations, cotyledons, leaves in various age groups

Recent botanical literature provides more and more information on the details of leaf anatomy in various taxonomic groups, including Boraginaceae; these traits are considered to be important diagnostic features for identifying the species status and clarifying relations, often support by molecular evidence (Dasti et al. 2003; Diane et al. 2003; Akçin & Baki 2007; Akçin et al. 2012, 2013; Hoyam & Kordofani 2012; Alwahibi & Bukhary 2013). There are a lot of works, reporting on the presence of adaptive traits related to the geographic distribution of plants or particular conditions in terms of soil or climate in the habitat and in the leaf microstructure (Selvi & Bigazzi 2001; Papp et al. 2011; Burkovskaya & Khrolenko 2015). However, these data are based solely on microscopic investigations of definitive plant individuals. Yet, leaf is an organ showing the highest adaptation ability of all vegetative organs; in most cases, changes in its histology can be clearly seen in its ontogeny (Nordhausen 1903; Schramm 1912; Alexandrov & Dzhaparidze 1930; Barykina & Gulenkova 1985 and many others). Furthermore, the range of age variability differs in representatives of various taxa and is determined by both internal driving forces and external environmental factors. For example, the heterogeneity of homonymous assimilatory organs in individuals of different age is quite distinct in species of *Mahonia* and *Berberis* (Berberidaceae), which are secondarily evergreen; this trait is established only from 3rd to 4th year of life in a seed-grown plant (Barykina & Chubatova 1980). The transformation from a deciduous to evergreen mode in juvenile plants is accompanied by the formation of polyfunctional hypoderm, missing in one-season leaves of seedlings and juvenile forms. This hypoderm ensures perennial existence of the organ through the enhancement of cambial activity in large vascular bundles and long-term (up to 1.5–2 years) superficial leaf blade growth, etc. Together with changes in the anatomy of leaves, gradually developing in ontogeny, structural and functional transformations of the same assimilatory organ so-called temporary heterogeneity

(Barykina & Gulenkova 1983) may take place. This functional shift and the corresponding histological transformation of cotyledons and leaves is quite a widespread phenomenon.

Subsequently, a question may arise: when in ontogeny do the key microstructure traits of assimilating leaves, characteristic of a particular species, emerge and stabilize and can one, based on that, identify the taxonomic identity of a plant at early ontogenetic stages, as early as in virginile period?

Our research included the study of leaf anatomy in virginile plants and those in the reproductive stage in nine species of Boraginaceae from national and foreign floras. The studied plants were of different life-forms and ecological backgrounds. The aim of the current study is to present a comprehensive description of leaf microstructure at different ontogenetic stages, to reveal the pathways of adaptive specialization and to assess the advisability of using anatomical traits of assimilatory organs in plant taxonomy.

Materials and methods

Herbaceous (mostly perennial and one annual) representatives of the family Boraginaceae were taken as material for our research: *Brachybotrys paridiformis* Maxim. ex Oliv., *Omphalodes verna* Moench, *O. linifolia* (L.) Moench, *Lindelofia stylosa* (Kar. & Kir.) Brand, *L. macrostyla* (Bunge) Popov, *Macrotomia euchroma* (Royle) Paulsen, *Symphytum ×uplandicum* Nyman, *Trichodesma incanum* (Bunge) A. DC., *Hackelia uncinata* (Benth.) C.E.C. Fisch. Sample plants for anatomical and morphological analysis were collected in the wild (Moscow region of Russia, Tadjikistan, Uzbekistan) and grown in cultivation (Botanic Garden of Lomonosov Moscow State University). Comparative morphological and anatomical analysis as well as ontogenetic methods were used. Anatomy of leaf apparatus in seedlings, sprouts, juvenile and definitive plants was studied in temporary freehand longitudinal and transversal (cross) sections, made with a razor blade through the middle of the leaf blade. Identification of substances and inclusions (cell wall lignin, lipids, e.g. cutin or oils, aleurone, etc.) was performed according to the reference book on microscopic techniques (Barykina et al. 2004). Epidermal cell configuration was described according to Zakharevich (1954). Mesophyll type was identified in compliance with recommendations of Vasilevskaya & Butnik (1981). Leaf and cotyledon microstructure was studied by means of light microscope Micromed 3. Anatomical drawings were made using a drawing device RA-1. For microphotographs, light microscope Axioplan-2 and cameras AxioCam MRc were used. The resulting images were edited in Adobe Photoshop CS6 whenever needed.

Abbreviations in figures. *chlrp* – chloroplasts; *clh* – collenchyma; *cut* – cuticle; *gh* – glandular hair; *h* – hair; *icsp* – intercellular spaces; *lep* – lower epiderm; *lv* – lateral vein; *mr* – midrib; *phl* – phloem; *prnch* – parenchyma; *psh* – parenchymal sheathing of vascular bundles; *pt* – palisade tissue; *rsc* – 'rosette' cells; *sh* – simple hair; *sp* – spongy parenchyma; *st* – stoma; *uep* – upper epiderm; *xl* – xylem.

Results

Brachybotrys paridiformis Maxim. ex Oliv. – perennial hemicryptophyte with a short horizontal rhizome, growing in shady mixed forests of the Far East and China. The plant occurs mostly on light soils such as loams (Gürke 1893; Popov 1953).
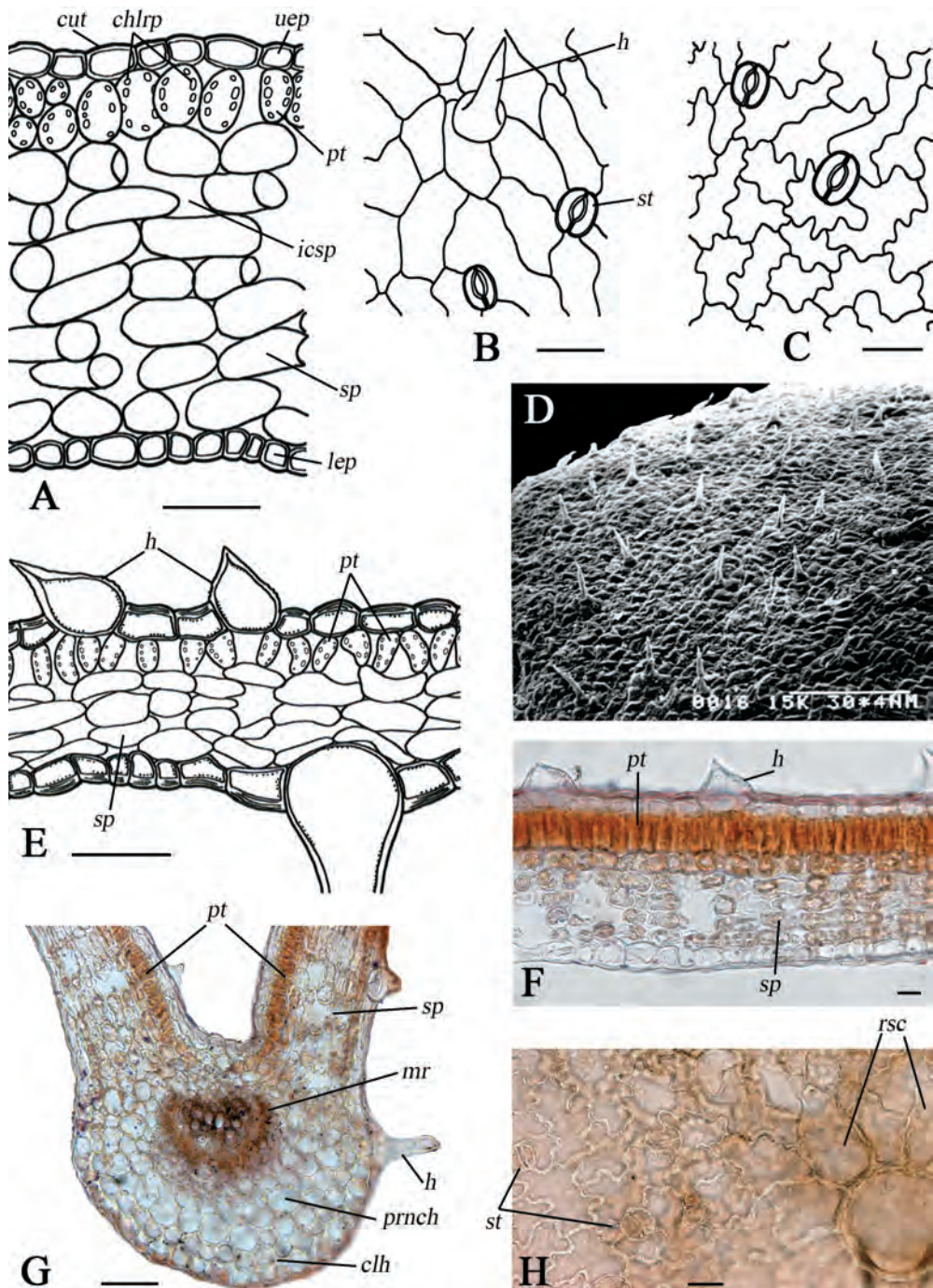
Figure 1. *Brachybotrys paridiformis.* Cotyledon: A – cross section of leaf blade (layout); B, D – upper epiderm; C – lower epiderm. The first leaf: E – cross section of leaf blade (layout). Definitive leaf: cross section through the middle of the leaf blade (F) and near the midrib (G); H – lower epiderm. Scale bars = 100 μm (A, D, G); 50 μm (B, C, E); 20 μm (F, H).

In protein-free seeds of *B. paridiformis* as well as in those of many Boraginaceae, large cotyledons, being the first leaves of the incipient shoot, are specialized as storage structures. At the time of germination, they are found to contain, along with uniform parenchyma, numerous oil drops, aleurone grains and small amounts of starch, fully formed procambial strands located at the
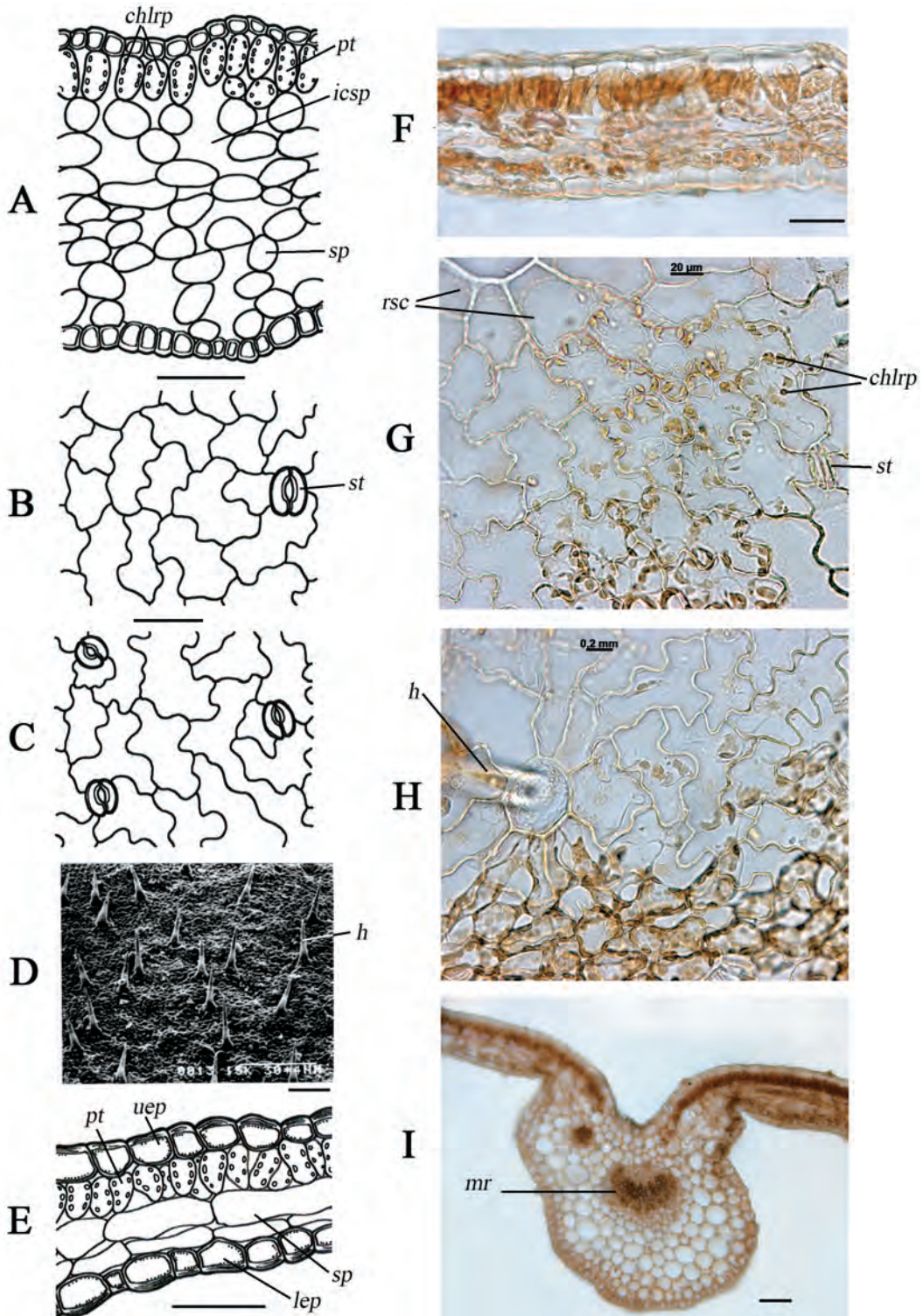
Figure 2. *Omphalodes verna*. Cotyledon: A – cross section of leaf blade (layout); B, D – upper epiderm; C – lower epiderm. The first leaf: E – cross section of leaf blade (layout). Definitive leaf: cross section through the middle of the leaf blade (F) and near the midrib (I); upper epiderm (G) and lower epiderm (H). Scale bars = 150 μm (D); 100 μm (A, I); 50 μm (B, C, E, F); 20 μm (G, H).

place of future midribs. No stomata or trichomes were observed. After the seedling is released from the seed envelope, in the course of subsequent cotyledonary germination (Barykina & Alyonkin 2017), the cotyledons, now able to photosynthesize, acquire a typical leaf-like structure. Anatomical transformations involve the epidermal tissue complex, mesophyll, veins, etc.

Cotyledon blades in *B. paridiformis* are fleshy (430–450 µm thick) (Fig. 1A), amphistomatic, pubescent on the upper surface and on the sides; pubescence uniform, scarce, scabrous; hairs one-celled, short, subulate, sessile and the 'rosette' cells at their bases are poorly expressed (Fig. 1D). Epidermal cells on the upper side (Fig. 1B) are slightly elongated longitudinally, flattened out, their anticlinal walls being somewhat wavy; on the lower side they are large and undulate (Fig. 1C). Stomata anomocytic, guard cells located in line with epidermal cells. In the lower epiderm, stomatic clefts are oriented along the blade, while in the upper one they do not seem to have any specific pattern of location. The number of stomata ranges from 27 (upper epiderm) to 38 (lower epiderm) stomata per mm^2 on average. The median vein (midrib) contains one to two bundles. The mesophyll is found to be dorsiventral, with one, less often two layers of relatively short loosely connected palisade cells; the spongy parenchyma consists of 7 layers of rounded and lobed cells. The palisade coefficient is found to be low (around 10–15%); chloroplasts are large.

Juvenile leaf blade (Fig. 1E) is amphistomatic, 2 to 2.5 times thinner (100–170 µm) than that of the cotyledon, with a thicker layer of smooth cuticle on the lower side. In juvenile, as well as in immature and mature reproductive plants, both surfaces of leaf blades are pubescent, differently from assimilating cotyledons in the seedlings. Trichomes of two types are found: simple subtended and sessile ones; both of them having bubble-like bases. Juvenile and definitive leaves have solitary stomata in their large-celled upper epiderm (the stomata concentrated mostly near the blade top); in the lower epiderm their number doubles (66 per mm^2). The blades of the first and subsequent leaves are dorsiventral (Fig. 1F, H), with distinctly differentiated single layer of palisade cells and from 3 to 5 layers of spongy parenchyma cells with pronounced network of intercellular spaces. The palisade coefficient reaches 32%. The midrib, consisting of a single vascular bundle, is much larger than lateral veins and protrudes the lower side, the protrusion exceeding the blade thickness by 1.5–2 times (Fig. 1G). Near the midrib, the mesophyll is gradually being replaced by colorless parenchyma, its two subepidermal layers resembling a collenchyma-like tissue.

Omphalodes verna Moench, a perennial stoloniferous polycarpic plant with a short rhizome, populating humid broadleaf forests in the lower montane belt, at rocky gorge slopes under trees, along river valleys among shrubs on rich, loose, slightly alkaline soils. The plant occurs in the southeastern Alps and the Romanian Carpathians (Popov 1953; Hege 1972).

Blades of assimilating cotyledons are fleshy (350–380 µm thick) (Fig. 2A), amphistomatic, pubescent on the upper side only. The pubescence is uniform throughout the entire surface, scarce, scabrous, of unicellular subulate (straight) sessile trichomes (Fig. 2D). The 'rosette' cells are poorly expressed. In paradermal sections, the adaxial epiderm cells (Fig. 2B) are elliptic in shape, elongated, their anticlinal walls flexuous to wavy, whereas on the adaxial side these cells are flattened and elongated, with massively flexuous to wavy anticlinal walls (Fig. 2C). Stomatal complexes are anomocytic, less often anisocytic, the guard cells together with subsidiary cells are slightly raised above the ground epidermal cells. Stomatal apertures on both sides of the blade have no particular orientation pattern. On average, there are 26 stomata per mm^2 on the adaxial and 62 on the abaxial side. The mesophyll is clearly differentiated into the palisade tissue (one

layer of short columnar cells and 8 to 9 layers of tangentially elongated rounded cells of spongy parenchyma, forming the net of intercellular spaces which are larger than those in *B. paridiformis*. Palisade coefficient 16%. Vascular bundles collateral, rounded in shape, fully submerged in the mesophyll. The midrib includes two vascular cords brought together.

The first leaf is found to be amphistomatic (Fig. 2E), four times thinner than the cotyledons (80 µm), both sides of it covered by unicellular, subulate, subtended trichomes of various length. Outer tangential walls of the upper and lower epiderm cells are markedly thickened. Stomata, small in number on the adaxial side (near the tip, 10 stomata per mm^2), account for average 108 per mm^2 on the abaxial side. The guard cells are in line with the ground epidermal cells or slightly raised. The mesophyll is dorsiventral. It comprises one row of palisade tissue and 2–3 layers of loosely arranged funnel- and dumbbell-shaped spongy tissue cells. Palisade coefficient 40%. The midrib's vascular bundle in cross section is larger than the lateral ones and is slightly protruding underneath. Towards the xylem, there is a subepidermal four- to five-layered site of collenchyma-like parenchyma.

The leaf blade in a mature (reproductive) plant is thin (Fig. 2F), amphistomatic, with scarce pubescence of short, pressed-down, unicellular trichomes. Stomata are concentrated mainly in the abaxial epiderm, yet their density is relatively moderate; guard cells slightly raised. Ground epidermal cells are larger upwards (Fig. 2G, H). In projection, they are flattened out, their walls flexuous. Mesophyll dorsiventral. Palisade tissue cells are short and funnel-shaped. Spongy parenchyma consists of 4–5 layers of lobed cells, markedly decreasing in size at the border with the lower epiderm. Palisade coefficient 40%. The midrib is of 2–3 fused vascular tissue strands, its protrusion 4 to 5 times thicker than the leaf blade (Fig. 2I). Near it, the palisade tissue zone interrupts and is replaced by colorless, thin-walled parenchyma cells, just like in case of the spongy tissue in the lower part. Lateral bundles are fully submerged in the mesophyll.

Omphalodes linifolia (L.) Moench, contrary to the previously described species, is a tap-rooted annual plant (Table 1). It occurs in southwestern Europe and northern Africa and predominantly populates dry habitats with well-drained soils.

The cotyledons are flat (Fig. 3A), with sparse short trichomes on the upper surface and margins of the blade, amphistomatic. Epidermal cells on both sides (Fig. 3B, C) are rectangular in cross section, with convex outer walls; in paradermal section, they are found to be flattened out, with flexuous anticlinal walls. Stomata small, slightly sunken. Mesophyll dorsiventral, with one layer of relatively short palisade cells with minute chloroplasts. Palisade coefficient about 20%. The spongy parenchyma is made of large round to lobed cells constituting 5–7(9) layers and penetrated by 14 to 15 small-sized vascular bundles, the one consisting of two strands is median.

The first leaf blade is thinner than the cotyledon blade (Fig. 3D). In cross section, one can note the adaxial epiderm (Fig. 3F, G) due to larger size of its cells with convex outer tangential walls, presence of sparse hairs, subtended at base by a one-layered 'rosette' of cells with transversely striate walls (Fig. 3H). In paradermal section, the epidermal cells are large, flattened out, with flexuous walls. Stomata anomocytic, their density is relatively low. Mesophyll isolateral-palisade. The palisade tissue is presented by one row on both sides, but its cells on the upper side are taller and are more closely brought together. The spongy parenchyma of three to four cell layers has a net of smaller-sized intercellular spaces; around numerous (over 25 in cross section) vascular bundles it seems to be replaced by parenchymal bundle sheathing. Below, near the midrib, the
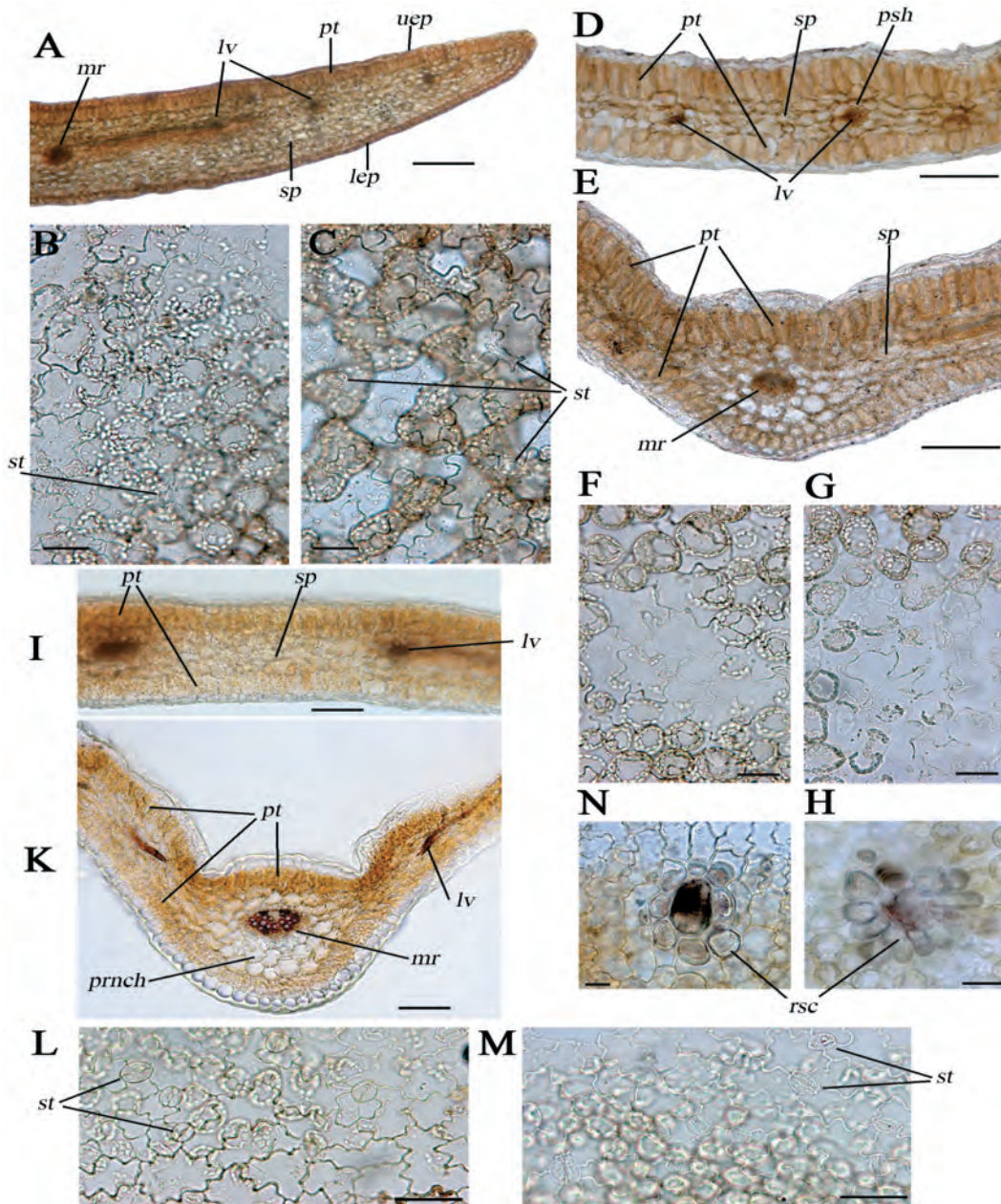
Figure 3. *Omphalodes linifolia*. Cotyledon: A – cross section of leaf blade; B, D – upper epiderm; C – lower epiderm. The first leaf: D – cross section through the middle of the leaf blade and E – near the midrib; upper epidermal cells (F) and cell of one-layered 'rosette' (H) with parallel layered (striate) structure; G – lower epidermal cells. Definitive leaf: cross section through the middle of the leaf blade (I) and near the midrib (K); upper epidermal cells (L) with 'rosette' cells (N) and lower (M) epiderm. Scale bars = 200 µm (A, D, E); 100 µm (B, C, I, K); 50 µm (F, G, H, L, M); 20 µm (N).

blade possesses a small protrusion, the palisade tissue is not interrupted in that area. The midrib comprises 2–3 fused strands of xylem and phloem elements, the latter armed with a layer of collenchyma-like tissue on the outside (Fig. 3E).

Definitive leaves are characterized by flexuous anticlinal and markedly thickened outer tangential walls of epidermal cells (Fig. 3 L, M). In these leaves, the 'rosette' cells (at the base of short simple

Table 1. Qualitative anatomical characteristics of leaf structure in plants of different age groups. * Mesophyll type (IP/DV): IP – isolateral-palisade, DV – dorsiventral.

Species	Life form • Ecological group	Cotyledons					First leaf					Definitive (mature) leaf					Geographic range	Climatic and edaphic conditions of the habitat
		Leaf blade thickness, µm	Mesophyll type (IP\DV) *	Number of palisade tissue layers	Number of spongy parenchyma layers	Palisade coefficient, %	Leaf blade thickness, µm	Mesophyll type (IP\DV) *	Number of palisade tissue layers	Number of spongy parenchyma layers	Palisade coefficient, %	Leaf blade thickness, µm	Mesophyll type (IP\DV) *	Number of palisade tissue layers	Number of spongy parenchyma layers	Palisade coefficient, %		
Brachybotrys paridiformis	Perennial hemicryptophyte with a short horizontal rhizome • Forest mesophyte	430–450	DV	1–2	7	10–15	100–170	DV	1	3–5	32	120–160	DV	1	3–5	32	Far East (Ussuriland, Sikhote Alin), China (Manchuria), Korean peninsula	Shady broadleaf forests, river valleys. Light soils, loams.
Omphalodes verna	Hemicryptophyte with a short rhizome • Forest mesophyte	350–380	DV	1	8–9	16	80–100	DV	1	2–3	40	140–160	DV	1	4–5	40	Southeastern Alps, Romanian Carpathians	Humid broadleaf forests in the lower montane belt up to 1300 m. Along river valleys among shrubs and herbaceous plants on rich, slightly alkaline soils
Omphalodes linifolia	Tap-rooted therophyte • Mesoxerophyte	400–420	DV	1	6 (9)	20	350–360	IP	2	3–4	50	225–230	IP	2	3–4	50–55	Southwestern Europe (France, Spain, Portugal), northern Africa (Algeria)	Dry habitats and semi-dry sun-lit habitats. Places with loose well-drained soils
Lindelofia stylosa	Tap-rooted hemicryptophyte with a polycephalous caudex • Mesoxerophyte	300–320	DV	2	12–14	20–25	375–380	IP	2	5	50	200–280	IP	3	3–4	70	Central Asia (Tien Shan, Pamir-Alay), Himalayas, Tibet	On rocky slopes, at temporarily moist habitats, alpine deserts. In the middle and upper (subalpine) montane belt at 1700–4500 m.

Species	Life form																Distribution	Habitat
Lindelofia macrostyla	Tap-rooted hemicryptophyte with a polycephalous caudex • Mesoxerophyte	310–320	DV	1–2	6	20	180–200	IP	1–2	5–6	30	–	IP	–	–	–	Central Asia (western Tien Shan, Pamir-Alay), Iran, Afghanistan, Pakistan	Semi-savannahs, subalpine meadows, sagebrush deserts. On rocky and stony slopes and glidders. In the lower or middle montane belt at altitudes of 700–3400 m.
Macrotomia euchroma	Hemicryptophyte with a polycephalous caudex and a thick taproot • Mesoxerophyte	900–1050	DV	2–3	15–19	30–35	360–380	IP	2–3	4–5	50	220–347	IP	2(3)	5–6	50–55	Central Asia (Tien Shan, Pamir-Alay), Iran, Afghanistan, Pakistan, Himalayas, western Tibet	In the belt of subalpine meadows and forb steppes, in alpine deserts. On rocky and stony slopes and glidders, along river valleys. In the upper montane belt at altitudes of 2800–4400 m.
Symphytum × uplandicum	Fibrous-rooted hemicryptophyte or geophyte with a short rhizome. • Sciophytic hygromesophyte	650–700	DV	1	11	16	100	DV	1	3–4	40	166–183	DV	1	6–7	30–35	Europe, North America, Middle Russia (Moscow city and region)	On plains in wet meadows, marshy areas, along rivers and streams, at wastelands, near dwelling areas
Trichodesma incanum	Hemicryptophyte with a robust branching rhizome • Mesoxerophyte	720–750	IP	2(3)	20	15–20	230–240	IP	2	4–6	50	180–220	IP	2	4–5	55	Central Asia (western Tien Shan, Pamir-Alay, Kopet Dagh), Iran, Afghanistan, Pakistan	In the belt of semi-savannahs and deserts on rocky and stony slopes, In lower and middle montane belt at altitudes of 600–2900 m.
Hackelia uncinata	Hemicryptophyte with a short rhizome • Sciophytic hygromesophyte	160–175	DV	1	5–6	15–20	150–165	DV	1	3	35–40	–	–	–	–	–	Asia (from Kashmir to Nepal, western China)	Montane forests at 2400–3500 m. Prefers moist soils.

trichomes) are found to have evidently striate walls (Fig. 3N); the palisade coefficient of isolateral-palisade mesophyll is high, around 50–55% (Fig. 3I). The spongy parenchyma is composed of only 3 to 4 layers of rounded and lobed cells. The midrib comprises two bundles; its protrusion exceeds the blade thickness 1.5–2 times (Fig. 3K).

Lindelofia stylosa (Kar. & Kir.) Brand is a tap-rooted polycarpic plant with a polycephalous caudex. It is widespread in the belt of microthermal steppes, alpine deserts of Central Asia, growing along rocky slopes, in temporarily moist habitats (Zakirov 1961; Chukavina 1984).

The seedling's (Fig. 4A) cotyledon blades are slightly broadened near the midrib, amphistomatic, with short accumbent unicellular subulate trichomes. The guard cells of the stomata are on the same level as the other epidermal cells; doubled stomata can be found. The ground epidermal cells on both sides have straight or slightly wavy anticlinal walls. Mesophyll dorsiventral, with two layers of relatively short palisade cells, oriented perpendicular to the surface, and 12 to 14 layers of tangentially elongated slightly lobed spongy parenchyma cells. Palisade coefficient 20–25%. The palisade character of mesophyll is not pronounced near the midrib. Here, the mesophyll is uniform, of thin-walled rounded cells with multiple minute chloroplasts and starch grains. All veins both median and lateral (exceeding 20 in number) have large-celled parenchymal sheaths, their cells lacking chloroplasts.

The first leaf's blade is rather fleshy (Fig. 4B). Its both sides are covered by long, simple, subulate trichomes subtended by 8–10-celled 'rosettes' at the base (Fig. 4E). The ground epidermal cells are large, with thickened outer tangential walls and flexuous anticlinal ones (Fig. 4D). The stomata, predominantly anomocytic, slightly sunken, large in number, are differentiated in the lower epiderm. The leaf tends to be isolateral. Mesophyll dorsiventral, possessing two layers of tall palisade cells on the adaxial side and five layers of spongy parenchyma, furthermore, minute cells of the lowermost layer of spongy parenchyma, adjacent to the epiderm, look like short palisade cells. They compose small groups, separated from each other by substomatal chambers. The palisade coefficient is very high, about 50%. In the midrib area, the blade is slightly concave on the upper side and broadened into a small projection on the lower side; mesophyll appears to be replaced by thin-walled rounded parenchyma cells. The midrib includes 2–3 strands of vascular tissue (Fig. 4C).

In definitive leaves, the flattened-out shape of ground epidermal cells is retained (Fig. 4H) together with wavy anticlinal walls and dense pubescence. Mesophyll isolateral-palisade, including 1–2 layers of palisade cells on the adaxial side and one layer on the abaxial (Fig. 4F). The spongy parenchyma consists of 3–4 layers of lobed cells, situated parallel to the surface. Palisade coefficient is 70%. The midrib most often possesses a large solitary collateral vascular bundle (Fig. 4G), in which the vascular tissues form a broad arch; sometimes, a small bundle may be located next to it. The midrib protrudes markedly on the lower side (the protrusion is 3–3.5 times thicker than the leaf blade).

Lindelofia macrostyla (Bunge) Popov resembles *L. stylosa* in its life form. The species is known to grow on rocky slopes in the middle and upper (subalpine) montane belt of Central Asia (Zakirov 1961; Chukavina 1984).

The pubescence of the flat cotyledons (Fig. 5A) is of sparse sticking out or partially appressed hispid type; trichomes are subtended by small 'rosettes' of 4–6 cells. Ground epidermal cells have
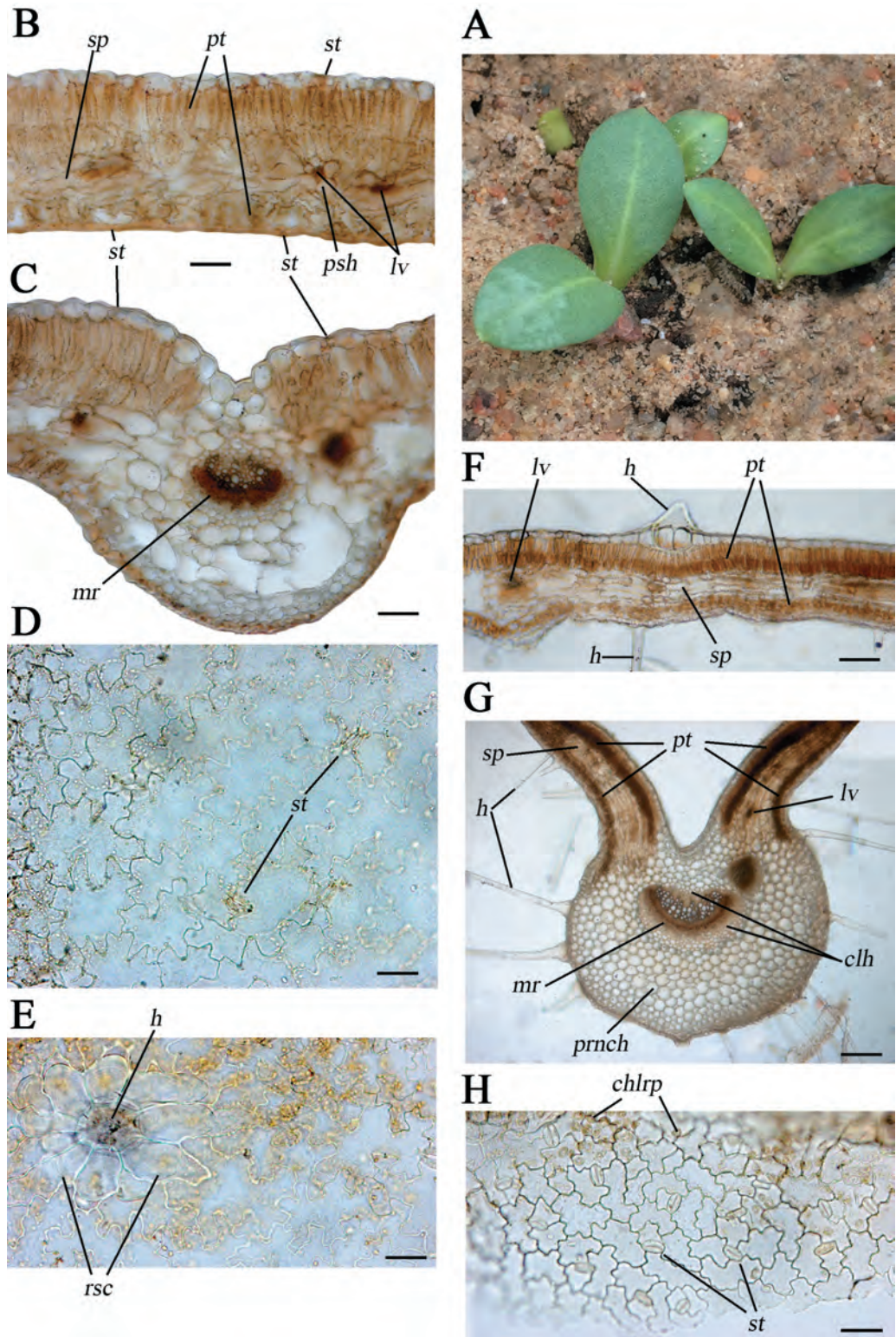
Figure 4. *Lindelofia stylosa*. A – seedling. The first leaf: cross section through the middle of the leaf blade (B) and near the midrib (C); upper epiderm (D) and lower epiderm (E). Definitive leaf: cross section through the middle of the leaf blade (F) and near the midrib (G); H – upper epiderm. Scale bars = 200 µm (G); 100 µm (B, C, F); 50 µm (D, E, H).
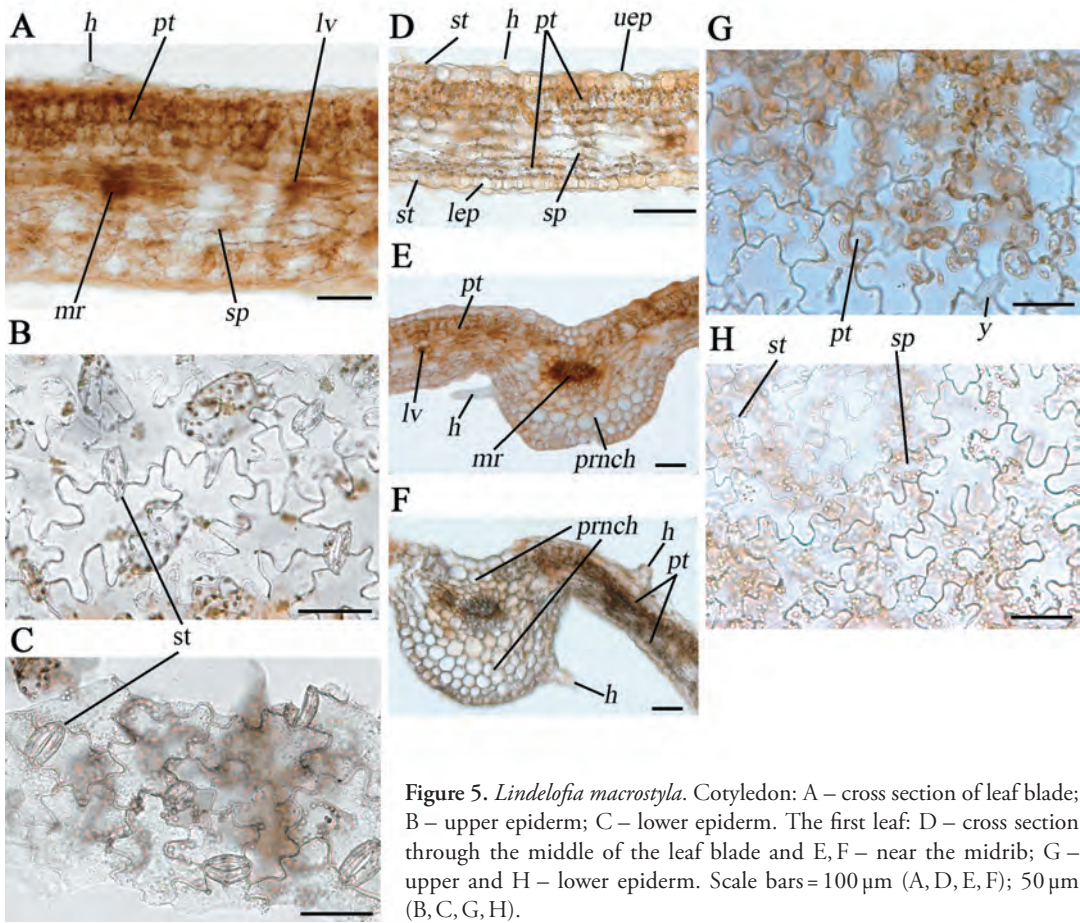
Figure 5. *Lindelofia macrostyla*. Cotyledon: A – cross section of leaf blade; B – upper epiderm; C – lower epiderm. The first leaf: D – cross section through the middle of the leaf blade and E, F – near the midrib; G – upper and H – lower epiderm. Scale bars = 100 μm (A, D, E, F); 50 μm (B, C, G, H).

rather poorly thickened outer walls; in paradermal section, they are flattened out with flexuous to wavy walls (Fig. 5B, C). Leaf blades amphistomatic. Stomata anomocytic, less often anisocytic; guard cells are at the same level with the ground cells. Stomata are less on the adaxial side (76 per mm^2) than on the abaxial side (82). Mesophyll dorsiventral: palisade tissue of 1–2 layers, palisade cells short, loosely arranged; spongy parenchyma of 6 layers, made of lobed cells and large intercellular spaces; palisade coefficient is about 20%. The midrib consists of 2–3 bundles and lateral veins possess bundle sheaths. All vascular bundles are coplanar, closer to the adaxial side.

The first leaf's blade is relatively thin (Fig. 5D), both sides of it are covered by one-celled long (their length exceeding the blade thickness 5–6 times) simple trichomes subtended by 'rosettes' of 8 or more cells. Ground epidermal cells on both sides are large, flattened out with deeply flexuous anticlinal walls (Fig. 5G, H). Stomata anomocytic or hemiparacytic, their density is greater than in the cotyledons, equaling on average 132 per mm^2 in the adaxial epiderm and 109 per mm^2 in the abaxial one. The mesophyll is differentiated into one- to two-layered palisade tissue and five- to six-layered spongy parenchyma, with its subepidermal cells rather small, tightly connected, similar to those of the palisade tissue in shape and orientation, i.e. the leaf tends to becoming isolateral. Palisade coefficient is about 30%. In the midrib (of 2–3 bundles) area, a pronounced protrusion is present (Fig. 5E, F), where the mesophyll is interrupted and replaced by thin-walled cells of uniform parenchyma.
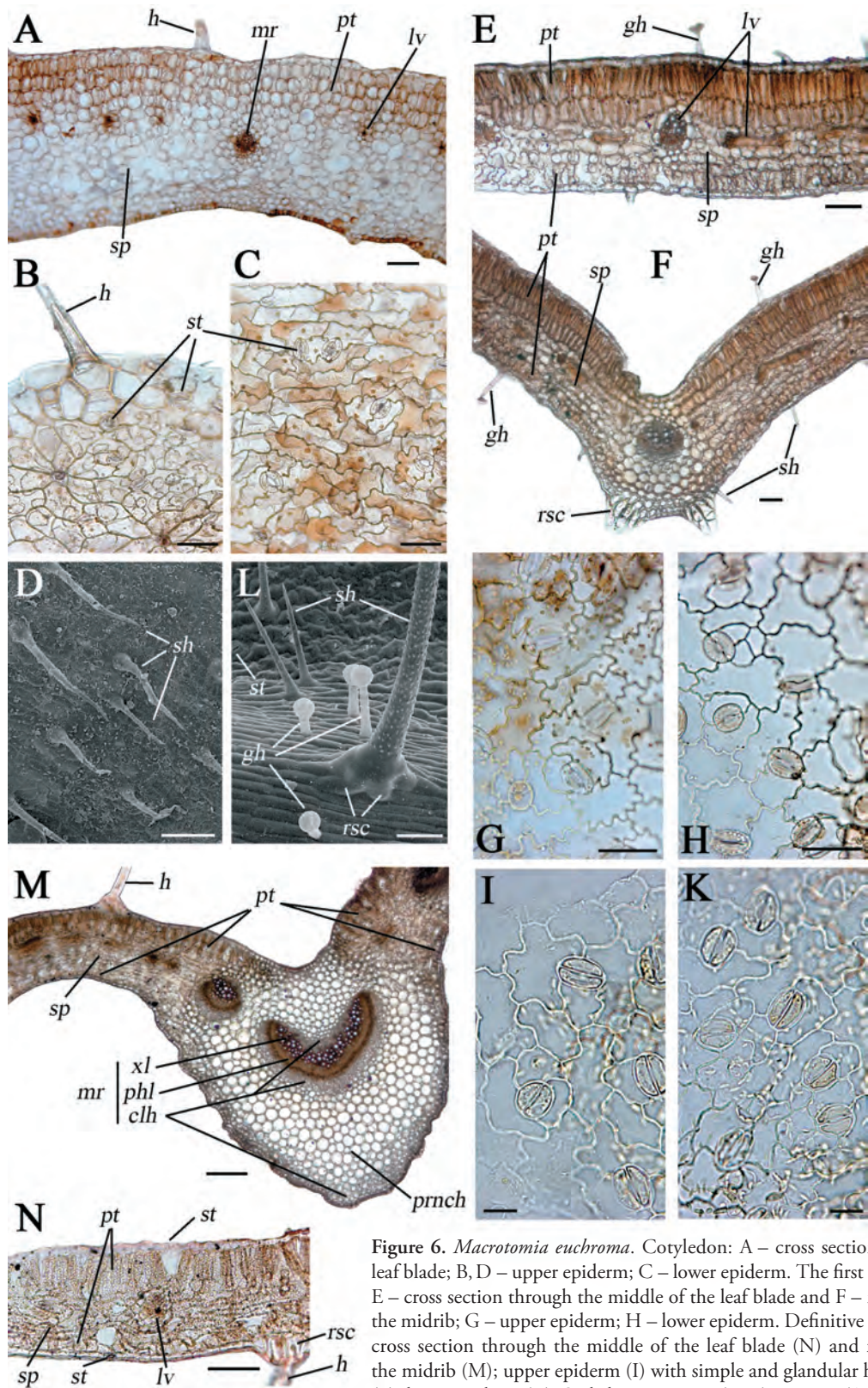
Figure 6. *Macrotomia euchroma*. Cotyledon: A – cross section of leaf blade; B, D – upper epiderm; C – lower epiderm. The first leaf: E – cross section through the middle of the leaf blade and F – near the midrib; G – upper epiderm; H – lower epiderm. Definitive leaf: cross section through the middle of the leaf blade (N) and near the midrib (M); upper epiderm (I) with simple and glandular hairs (L); lower epiderm (K). Scale bars = 300 µm (D, L); 200 µm (A, M); 100 µm (B, C, E, F, N); 50 µm (G, H); 20 µm (I, K).

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In definitive leaves of *L. macrostyla,* just like in those of *L. stylosa*, isolateral-palisade mesophyll type is better expressed.

Macrotomia euchroma (Royle) Paulsen is a perennial hemicryptophyte with a thick (up to 2 cm) staining taproot and polycephalous caudex. It occurs on rocky slopes in the middle and upper (subalpine) montane belt (Zakirov 1961; Chukavina 1984).

Fleshy cotyledons (Fig. 6A) in this species are pubescent, amphistomatic, the stomata are slightly raised. Ground cells of the upper epiderm are larger than those of the lower; thick-walled trichomes, subtended at the base by a 'rosette' of small cells (Fig. 6B, C) are present. Mesophyll dorsiventral, with 2–3 layers of short, wide palisade cells. Palisade coefficient about 30–35%. The spongy parenchyma (of 15–19 layers) is represented by quite large round cells and a net of small intercellular spaces. Vascular bundles are small, up to 25 in number in the blade cross section, separated from the mesophyll by parenchymal sheaths. Midrib of one or two bundles.

The first leaf's blade differs greatly from the cotyledons by its abundant pubescence and diverse trichomes (Fig. 6E). Along with relatively short subulate thick-walled trichomes rounded at the base, there are robust long hairs, subtended by 'rosettes' of two layers of rounded cells (primarily on the adaxial side), as well as glandular trichomes of one- to two-celled stalks and heads. The ground epidermal cells in cross section appear to have thick outer tangential walls, in paradermal section they are flattened out, with flexuous walls (Fig. 6G, H). Numerous non-sunken stomata, anomocytic or rarely hemiparacytic or anisocytic, are scattered throughout. Mesophyll isolateral-dorsiventral; on the adaxial side, there are 2–3 layers of tightly brought together narrow palisade cells, adjacent to parenchymal sheaths of vascular bundles or to rounded and lobed cells of four- to five-layered spongy parenchyma. The latter is being replaced by short, loosely connected palisade cells. Palisade coefficient about 50–55%. The midrib consists of two vascular bundles fused into a single strand (Fig. 6F). In this area, the blade forms a small protrusion on the lower side, where the mesophyll is interrupted and replaced by colorless parenchyma.

In a definitive plant (Fig. 6N), leaves differ from the first seedling's leaf and subsequent juvenile ones mainly by qualitative factors. The ground epidermal cells are smaller in size (Fig. 6I, K); the number of stomata per mm^2 is growing, among the trichomes, the long hispid ones become the most numerous, and glandular trichomes are very few (Fig. 6L); the midrib becomes more robust (Fig. 6M), xylem and phloem elements being reinforced with collenchyma cells from the outside. This collenchyma-like tissue in a small amount can differentiate subepidermally at the periphery of the protrusion.

Symphytum × uplandicum Nyman is a perennial fibrous-rooted hemicryptophyte or geophyte with a short rhizome. The species is quite widespread in Europe and North America; only very few locations of the plant in Middle Russia are know (Moscow city and region). The plant grows in wet meadows, marshy areas, along rivers and streams (Hege 1972; Pawłowski 1972; Tikhomirov et al. 1999).

The assimilating cotyledon blades are of moderate thickness (650–700 µm), dorsiventral, amphistomatic, covered by short bristle hairs (Fig. 7A). They are unicellular, more or less equal in size, sessile, subulate (straight and hooked). In paradermal section, ground cells of the upper epiderm are flattened out and elongated, their walls are flexuous and wavy. Stomata anomocytic, elliptic in shape, rather large (80 µm long, 50 µm wide), located at the same level with the ground epidermal cells scattered with no particular pattern. On the adaxial surface, the average
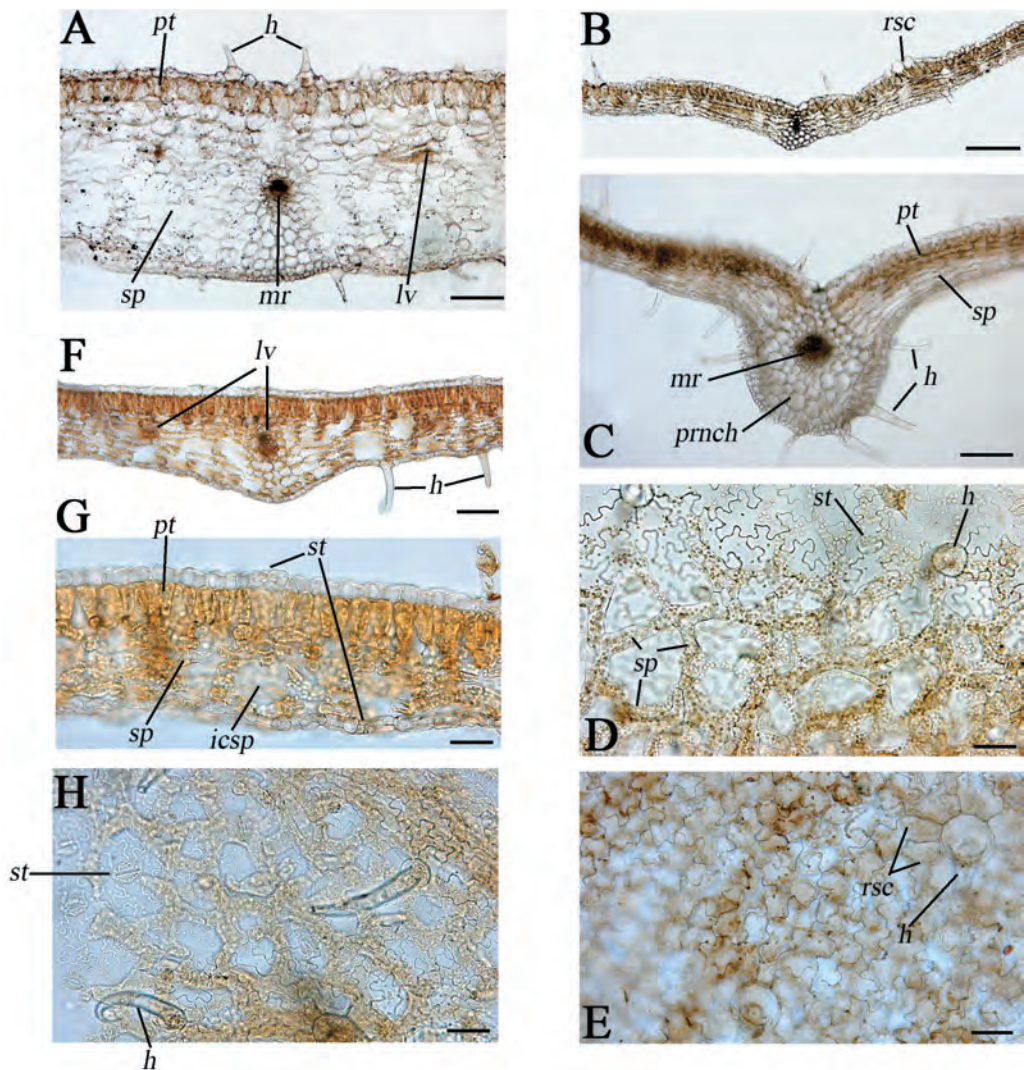
Figure 7. *Symphytum × uplandicum*. Cotyledon: A – cross section of leaf blade. The first leaf: B – cross section through the middle of the leaf blade and C – near the midrib; D – upper epiderm; E – lower epiderm. Definitive leaf: cross section through the middle of the leaf blade (F, G); lower epiderm (H). Scale bars = 200 μm (A, B, C); 100 μm (F, G); 50 μm (D, E, H).

density of stomata is 35 per mm², on the abaxial surface 50 per mm². Mesophyll dorsiventral: one layer of short palisade cells and 11 layers of spongy parenchyma with the net of small and large intercellular spaces. Palisade coefficient is 16%. The midrib vascular bundle is completely submerged in the mesophyll, so are the lateral ones.

The seedling's first leaf blade is almost 6 times thinner than that of the cotyledon (100 μm versus 650 μm respectively) (Fig. 7B), pubescent mostly on the upper side; subsequent leaves have trichomes on both surfaces. Two types of hairs are identified: short simple sessile trichomes and long subtended ones. Stomata anomocytic; the guard cells are slightly raised above the ground epidermal cells (Fig. 7D, E). Compared to the cotyledons, the number of stomata on the adaxial surface of the first leaf is decreased to 16 per mm² and increases to 87 per mm² on the abaxial surface. The mesophyll is clearly differentiated into the palisade tissue (one cell layer) and spongy
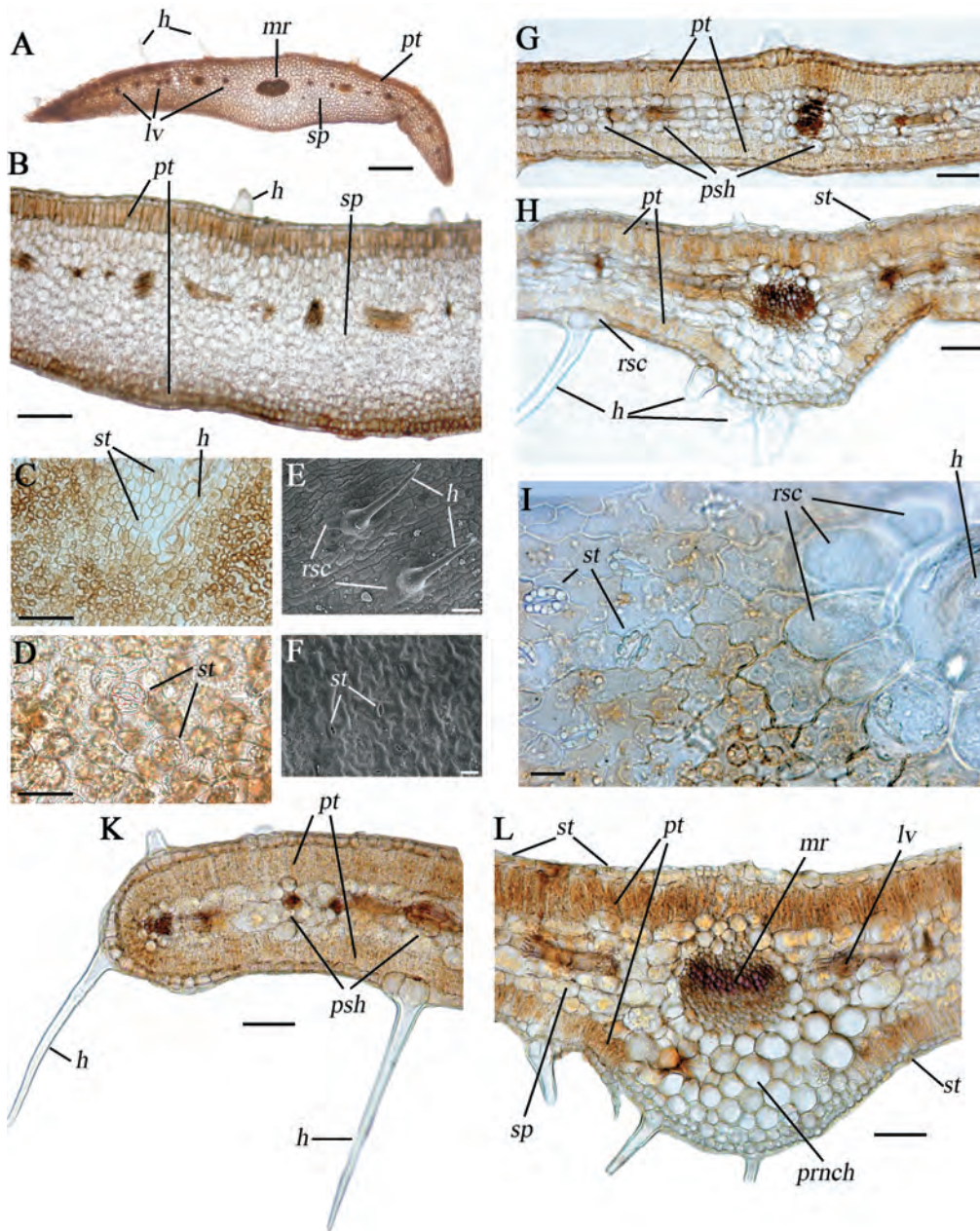
Figure 8. *Trichodesma incanum.* Cotyledon: A, B – cross section of leaf blade; C, E – upper epiderm; D, F – lower epiderm. The first leaf: G – cross section through the middle of the leaf blade and H – near the midrib; I – lower epiderm with 'rosette' cells. Definitive leaf: cross section of leaf margin (K) and near the midrib (L). Scale bars = 500 µm (A); 200 µm (B); 100 µm (E, G, H, K, L); 50 µm (C, D); 30 µm (F); 20 µm (I).

parenchyma (3–4 layers of tangentially elongated cells) with a net of large intercellular spaces. The palisade coefficient reaches 40%. Near the midrib and the largest lateral veins, protruding below, the mesophyll is replaced by parenchyma (Fig. 7C).

The definitive leaf blade is thin (Fig. 7G), both sides of it are covered by short subulate hooked simple one-celled trichomes, both sessile and subtended (Fig. 7H). The blade is amphistomatic; on the abaxial side the guard cells are slightly raised. Ground epidermal cells are larger on the

upper side. They are flattened out in projection, with flexuous walls. Mesophyll dorsiventral. The palisade tissue is one-layered, the spongy parenchyma has 6–7 cell layers with a net of large intercellular spaces (Fig. 7F). Palisade coefficient is 30–35%. The midrib most often contains several vascular bundles (a large central collateral bundle and 3–4 smaller ones). It clearly protrudes below (the protrusion exceeds the leaf blade thickness 7–8 times). In the midrib area, the palisade tissue interrupts, replaced by colorless parenchyma, minute-celled on the upper and large-celled on the lower side. Lateral veins also protrude on the abaxial side, their protrusion exceeding the blade thickness 2–3 times on average.

Trichodesma incanum (Bunge) A. DC. is a perennial hemicryptophyte with a strong branching rhizome. The species is distributed in Central Asia, where it grows on rocky slopes in the lower and middle alpine belt at 600–2900 m above sea level (Zakirov 1961; Chukavina 1984).

Cotyledons fleshy (Fig. 8A), slightly broadened in the middle, amphistomatic, with numerous trichomes, primarily on the adaxial side (Fig. 8E, F). Trichomes simple unicellular, subtended by the 'rosettes' of 6 cells, thick-walled, with remaining protoplasmic content. Stomata differ in size, often doubled, located with no particular pattern, somewhat sunken, anomocytic, hemiparacytic and anisocytic. In paradermal sections, epidermal cells are isodiametric and oblong with straight or slightly wavy walls (Fig. 8C, D). Mesophyll is isolateral-palisade. On the adaxial side, one or, less often two layers of narrow palisade cells containing multiple chloroplasts are differentiated. Smaller and shorter palisade cells conspicuously stand out on the abaxial surface of the cotyledons as well. Palisade coefficient 15–20%. The spongy tissue is composed of up to 20 layers of round thin-walled cells (Fig. 8B); the abundance of water-bearing parenchyma ensures the cotyledons to be succulent. Vascular bundles, which reach 40–50 in number near the middle of the blade, tend to be closer to the adaxial side. The midrib consists of two vascular bundles, brought close together or even completely fused. Just like numerous lateral bundles, the midrib bundles are surrounded by a parenchymal sheath, in which the cells are found to contain solitary chloroplasts.

The sprout's first leaf has a thinner blade than the cotyledons (Fig. 8G). Thick cuticle is quite pronounced and partially spread on the outer parts of epidermal cells' anticlinal walls. Multiple trichomes on the upper and lower sides are subtended by 'rosettes' of 8 or more large cells (Fig. 8I). In paradermal section, the ground epidermal cells are flattened out, with flexuous walls. Anomocytic stomata, large in number, are not sunken. Mesophyll isolateral-palisade: each side of the leaf blade has a row of palisade parenchyma of tall cells, between them being 4–6 layers of spongy parenchyma. Palisade coefficient is about 50%. The mesophyll is interrupted near the midrib of 2–3 vascular bundles only abaxially, where it is replaced by large round thin-walled cells of colorless ground parenchyma, forming a small-sized projection (Fig. 8H). Numerous lateral vascular bundles (up to 50 in cross section) are present.

Definitive leaves have the palisade coefficient of about 55% (Fig. 8K). Near the midrib, the mesophyll is sometimes interrupted not only on the lower, but also on the upper side of the blade (Fig. 8L).

Hackelia uncinata (Benth.) C.E.C. Fisch. is a perennial hemicryptophyte with a short rhizome. The species occurs in Asian montane forests at 2400–3500 m above sea level, preferably on moist soils.

Cotyledon blades are relatively thin, amphistomatic, with short subulate trichomes on them. Epidermal cells thin-walled, larger and with convex outer tangential wall; from the topside view, the cells look flattened out, with flexuous walls (Fig. 9A). Stomata anomocytic, somewhat
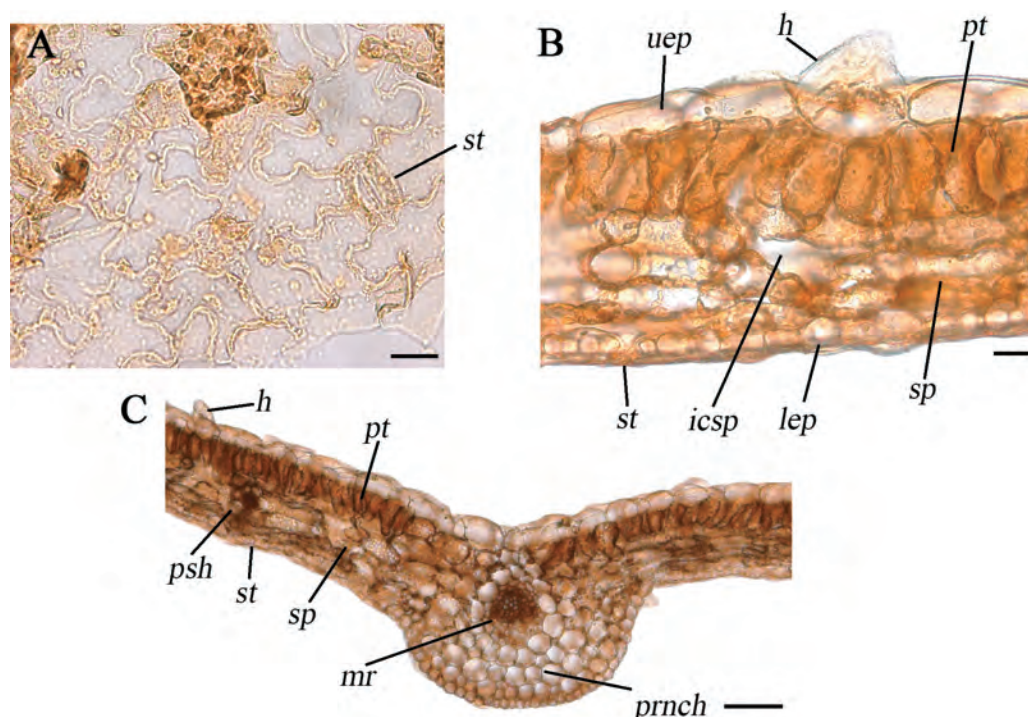
Figure 9. *Hackelia uncinata*. A – lower epiderm of the cotyledon. The first leaf: B – section through the middle of the leaf blade and C – near the midrib. Scale bars = 100 μm (C); 20 μm (A, B).

sunken. Mesophyll dorsiventral, with a single layer of narrow palisade cells and 5–6 layers of spongy parenchyma of rounded and lobed cells with large intercellular spaces. Palisade coefficient 15–20%. In the cross section of the cotyledon blade, there are over 20 vascular bundles; midrib of one to two bundles fully submerged in mesophyll.

In the sprout, the first leaf blade remains thin, amphistomatic, with dorsiventral mesophyll (Fig. 9B). Contrary to cotyledons, it is more densely pubescent; the trichomes have broadened bulb-shaped bases. The stomata appear primarily on the abaxial side; epidermal cells increase in size (an epidermal cell covers 2–3 palisade cells) and so do intercellular spaces in the spongy tissue; palisade coefficient seems to grow, too (up to 35–40%). The midrib is conspicuous (Fig. 9C), protruding on the abaxial side (the protrusion exceeds the leaf blade thickness 1.5 times); near the midrib, both palisade and spongy tissues are replaced by uniform parenchyma of rounded thin-walled cells.

Definitive leaves have not been studied due to absence of material for research.

Discussion

The comparative anatomical analysis demonstrated that the leaf-like structures emerging in succession in the plant ontogeny are not identical. This type of age variability bears the impact of climatic and edaphic conditions, which took place at the time of initiation, germination and further development and function of these structures. Thus, the thickest, fleshiest leaf blades of assimilating cotyledons, with 19–20 mesophyll rows within, are found in Central Asian desert and alpine mesoxerophytes *Macrotomia euchroma* (up to 1050 μm) and *Trichodesma incanum*

(750 µm) (Table 1). The former appears to have high palisade coefficient (up to 35%) coupled with dorsiventral type of mesophyll. In *T. incanum*, differently from other 8 studied species, the cotyledons have isolateral-palisade mesophyll, which is seemingly connected with higher solar radiation in the species' habitat (rocky slopes). Relatively thin (160 – 175 µm) cotyledon blades with six- to seven-layered mesophyll are found in the sciophytic hygromesophyte *Hackelia uncinata*, growing in Asian alpine forests on excessively moist soils. All species have amphistomatic cotyledons covered by simple hairs, differentiated mesophyll (1–3 rows of palisade tissue) and fully submerged veins (including the midrib). Collateral vascular bundles have parenchymal sheathing. No specialized supporting tissues are reported.

All things considered, the first leaves of sprouts and juvenile plants retain structure similar to that of assimilating cotyledons. However, the microstructure is different from that of both the cotyledons and subsequent definitive leaves. These age-related anatomical changes are species-specific. The blades appear to be thinner (from 80 µm in *Omphalodes verna* to 380 µm in *Macrotomia euchroma*), chiefly due to the reduction of spongy tissue rows (Table 1). At the same time, the palisade coefficient grows to 40–50% as a result of formation of more palisade tissue rows or palisade cell heights. In *O. linifolia*, *M. euchroma*, *Lindefolia stylosa*, *L. macrostyla* and *T. incanum*, the mesophyll is isolateral-palisade. The midrib can contain a single vascular bundle (*H. uncinata*), two (as in most species) or rarely three bundles. The midrib, together with large lateral veins, may protrude underneath to a various extent. The pubescence density increases; multiple subulate trichomes are subtended by well-expressed 'rosettes'; in *M. euchroma*, *L. stylosa* and *L. macrostyla*, transitory glandular trichomes, in addition to simple ones, composed of a one- to two-celled stalk and a head, are present. In plants which have reached the reproductive phase, the leaf blade thickness is almost the same as in virginile plants, except for large-leaved *Symphytum × uplandicum*, in which it is two times greater due to abundant spongy parenchyma (Table 1). All plants retain flattened out shape of ground epidermal cells and flexuous anticlinal walls and amphistomatic blade character throughout their ontogeny. In five species (out of nine studied), tending to populate open, well-lit and dry habitats, the mesophyll is found to be isolateral-palisade and stomata are small in size and great in number per mm^2 of the leaf surface. When the plant ages, the palisade coefficient grows up to 55–70% and the venation density increases as well as the size and development of the midrib and large lateral veins. Vascular strands in the midrib of alpine mesoxerophytes are supported by outer collenchyma. The collenchyma-like tissue is also differentiated subepidermally near the protrusion, where the mesophyll interrupts being replaced by colorless parenchyma.

Therefore, in studied Boraginaceae, considering the type of microstructure transformation of assimilating cotyledons and leaves in plants of various ages, consecutively emerging in ontogeny, one can define two developmental types, homoblastic and heteroblastic (Goebel 1928). In case of the former, age-related changes in anatomy are pronounced, often of qualitative character and appear at early ontogenetic and morphogenetic stages. The leaf blade type transforms (bifacial into isolateral in annual *O. linifolia* and perennial *L. stylosa*, *L. macrostyla*, *M. euchroma*, *T. incanum*), also, the expression of heliomorphic and xeromorphic traits increases. Similar age-specific changes in assimilating leaf-like organs are found in Central Asian petrophyte *Rindera tetrapsis* described by Butnik et al. (2009). In the latter, the differences are less pronounced, usually quantitative and gradual (other studied species). Sylvatic mesophytes *B. paridiformis* and *O. verna* and sciophytic hygromesophytes *S. × uplandicum* and *Hackelia uncinata* retain dorsiventral mesophyll type

throughout ontogeny, which correlates with their ecology (Table 1). These species grow in low light conditions and often high humidity in montane and lowland forests or river valleys. In the meantime, high alpine insolation causes early and pronounced mesophyll differentiation up to isolateral-palisade type as reported for fleshy cotyledons of the Central Asian hemicryptophyte *T. incanum*.

Thus, the comparative anatomical analysis of assimilating organs including cotyledons, first and subsequent juvenile and definitive leaves of studied Boraginaceae revealed a number of traits reflecting the adaptive strategy of each species to its habitat, even at early ontogenetic stages. Ecological groups of mesophytes, mesoxerophytes and sciophytic hygromesophytes are clearly defined. The most representative traits are: the structure of the epidermal complex, bifacial or isolateral leaf blade type, size of intercellular spaces, midrib protrusion, number of vascular bundles in the midrib, presence of collenchyma, etc. The study demonstrates that microstructure traits of assimilating organs can be used as significant diagnostic traits at the species level not only in mature, but also in virginile plants.

Acknowledgements

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