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The plasticity of anatomical structure and cell wall lignin in *Trapa natans* adaptation to nature flooding

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Summary

The experimental data of anatomical structure and lignin cytochemical study of *Trapa natans* (water chestnut) roots, submerged leaves and floating leaves were presented. It was established that the special anatomical variations of *T. natans* submerged organs promote to adaptation of plant to nature flood. The cytochemical method was used for the study of both distribution monolignols (syringil and guaiacyl) and also its relative content in cell walls of differ tissues. The localization of monolignols was analyzed with laser scanning microscope LSM 5 Pascal (Germany) and the content of monolignols was determined with Pascal program. It is established the differences of monolignols content in depend on organs and tissue. The studies encourage concept that under plant flood the monolignols of cell walls of submerged organs are involved in cellular and functional mechanisms of tolerance to nature flooding of plants.

Introduction

The study of morphological and anatomical signs of real hydrophytes and air-aquatic plants, including of *Trapa natans*, used for systematic of plants, for research of adaptation signs of plants to the flood and also for pharmacological activity of this plants (O'Neill, 2006; Prafulla Adkar et al., 2014). It is known that many air-aquatic plants including water chestnut (*T. natans*) are heterophyllous. It is known that submerged leaves and submerged roots of plants are characterized by specific anatomical and structural-functional features, including the decreased respiration and weak carbon feed that helps the submerged organs to adapt to the decline of sunlight in water, change of composition of light, content of CO_2 and O_2 in an aquatic environment (Armstrong et al., 1994; Vartapetian, Jackson, 1997; Jackson, 2008; Jackson, Colmer, 2005).

Lignin is one of the main structural polymeric cell wall of higher plants, fulfils a number of functions, among which mention should be made of their remarkable mechanical strength properties, wall impassability for water. Lignin is complex biopolymer of aromatic alcohols, which are synthesized in secondary cell walls. Lignin is highly branched and composed of cross-linked units, three monolignols: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) phenylpropanoid units (Boerjan et al., 2003). Lignin synthesis is depending from tissue type, organ and species (Monties, 1998; Fengel, Wegener, 1984). This biopolymer can to reduce speed of cell growth and participates in adaptation of plant to the stress**,** changing structure of cellular wall matrix, providing impassability of water and water solutions through walls and forming in an epidermis or other tissues

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a barrier to the pathogens. It is known that wall lignification intensify plant resistance from pathogens invasion (Moura et al., 2010). It is possible, that phenomenon is occurs because of lignin is characterized by hydrophobic feature, forming to hydrogen and covalently association between polysaccharides (Boerjan et al., 2003).

It is known that plants which expose to flood or submerged plants (hydrophytes) adapt to constant submerged conditions by the changes of morphology-anatomical structure and change of cell wall structure of epidermal tissue that is the first barrier between plant and water environment. Because the aim of our study was to carry out of the investigation the presence and distribution of individual monolignols in different cell walls and research of particular anatomical characteristics of submerged organs in *Trapa natans* air-water plant.

Materials and methods

Plant material. A research objects were submerged adventitious roots, submerged finely divided feather-like leaves and stems of water chestnut (*Trapa natans* L.) plants that grew on a depth up to 80 cm on the birch of the Rusanivsky channel (left Shore of Dnepr River, in Kiev). Floating and submerged leaves, and submersed adventitious roots and stems were collected at budding-flowering stage (beginning of July). The sun illumination [photosynthetic photon fluency rate (PPFR)] on water surface was 1500-1600 μmol quantum·m^{-2}·sec^{-1}, and on surface of the upper leaves of studied plants (about 8-10 cm below the water surface) was 10-12 μmol quantum·m^{-2}· sec^{-1}. PPFR was measured by the means of Light Meter Li-250 (USA, LI-COR).

Microscopical and cytochemical analyses. The middle part of floating leaves, submerged adventitious roots, and of needle-shaped particles of submerged dissected leaves were used for research**.** The sections of samples were selected from five plants, which had close identical size and close morphology, and then they were used for the light microscopy. Immediately after removal from water, samples were fixed on a birch in mixture of 5 % solution of paraformaldehyde and 2 % glutaraldehyde (1:1, vol) in a 0.5 *M* phosphate buffer, for 24 h, pH 7.2. Then the fixed material in laboratory terms was washed in buffer, dehydrated in ethanol and acetone, embedded in mixture an epon/araldite resin according to the standard method. Semithin sections (about 12 μm thickness) were stained with Schiff solution and by solution of safranine in accord with standard (Furst, 1979) and were studied on the light microscope (Axioscope, Carl Zeiss).

The cytochemical method of monolignols accordingly to Wuyts et al. (2003) protocol was used for the study of the present and the distribution syringyl and guaiacyl and also for detection of the relative content of these monolignols in cell walls. Middle part of swimming leaf, needle-like particles of submerged dissected leaf and transversal sections of adventious root (near 1 cm from apex) were hand cut and dipped in the staining solution of saturated (0.25%, w/v) diphenylboric acid-2-aminoethyl ester (DPBA) (Sigma) in deionized H_2O containing 0.02% (v/v) Triton-X-100 for 2-4 min at +25ºC; washed in H_2O, put in 0.05% solution of paraformaldehyde in phosphate buffer at +4ºC and then in laboratory samples were washed with such buffer and investigated on the laser scanning microscope (LSM 5 Pascal, Carl Zeiss, Germany). Complex DPBA–syringyl was excited at 340-380 nm, and fluorescence emission detected at 430 nm. Complex DPBA–guaiacyl was excited at 450-490 nm and fluorescence emission detected at 520 nm using an x 10, x 20 and x 40 objectives. Chlorophyll auto fluorescence was excited at 440 nm and fluorescent emission detected at 662 nm. Fluorescence intensity of monolignols and chlorophyll was measured in the cell walls as a function of emissions wave length using the PASCAL program (LSM 5, Carl Zeiss). The volume tissues in leaflets were measured with using ImageJ programs. Seven plants were used for the study. Five floating, five submerged leaves and five roots were used from each plant for anatomical and

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cytochemical investigations. We present the average fluorescence intensity in 30-40 cells of five floating, five submerged leaves and five roots. The cells were scanned three times in each organ. Values of results were expressed at the mean and standard errors, using Student's test ($P< 0.05$).

Results and discussions

Morphological and anatomical analysis of leaves and roots.

Plants of *Trapa natans* are one-year hydrophyte. The complete cycle of this plant ontogenesis survive about three months (June, July and August) in Ukraine. Plant of *T. natans* is characterized by the presence of developed floating (Fig. 1A), submerged leaves and adventitious submerged roots (Fig. 1B) at the stage of plant flowering. Floating leaves forms a rosette; leaflet is solid and triangular; the middle size of leaflet is 5.2 x 6.4 cm.

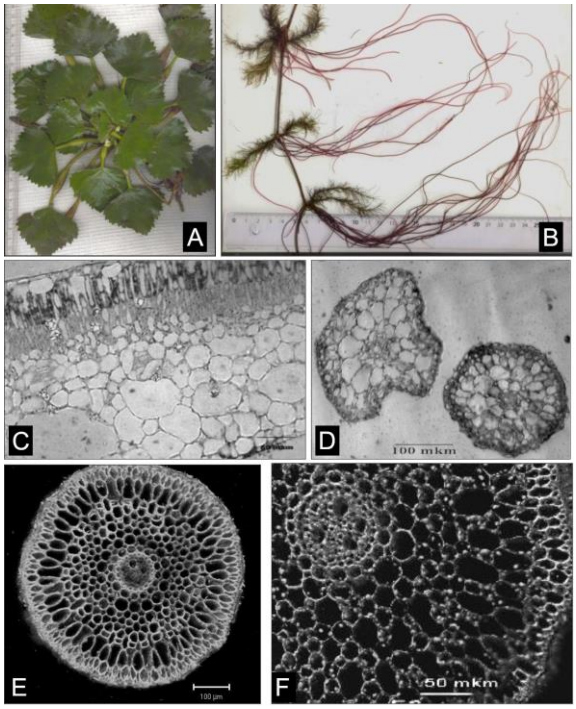

Fig. 1. General view of *Trapa natans* swimming leaves (a), submerged dissected leaves (green) and adventitious roots (b), and the sections through central plane of swimming leaf (c), submerged leaves (d) and submerged adventitious roots (e, f). Bars: C, F = 50 μm; D, E = 100 μm.

Two or four submerged dissected leaves are formed from each internodes of stem. Submerged green dissected leaves have feather-like shape. These leaves are situated oppositely against each other. The middle size of submersed dissected leaf is 53 ± 4.9 cm in long axis and 2.7 ± 0.3 cm− in short axis. Such leaves are consisted of long needle-like particles; the length of that comes up to 25 mm.

An adventitious lilaceous roots are formed in base of each stem node near submerged dissected leaves. These roots is very thin and have pinkish color, their length is from 7.5 to 31 cm; root hairs are absent. Away from node stem from 7 to 12 adventitious roots are formed (Fig. 1B). Morphology of floating and submerged leaves at flowering stage was like to that at vegetative stage (Nedukha, 2013)

Floating leaf. A dorsoventral structure is characterized for leaflets of floating leaves (Fig. 1C); including of bilamella palisade parenchyma, 6-9 layer of spongy parenchyma, aerenchyma (air cavities) between cells of 6-9 layer of spongy parenchyma and between adaxial epidermis and palisade cells, single-layered adaxial (with stomata) and abaxial epidermis. The middle number of chloroplasts in palisade cell is equaled 13 ± 2.7 on the section. Results of middle area these tissues in leaflets were next: adaxial epidermis – 6.5 %, abaxial epidermis – 6.7 %, palisade parenchyma – 38.5 %, spongy parenchyma – 47.9 %. It is necessary to note, that middle area of aerenchyma cavities was amount 11.9 ± 2.1 %. The size of cells in tissues of flooding leaves was like to that in vegetative stage (Nedukha, 2013).

Submerged leaf. Light-microscopic analysis of the transverse sections of needle-shaped particles dissected submerged leaf with rounded or slightly curved lateral shape is exposed unifacial, centric structure leaf (Fig. 1D), including one-layered epidermis, 4-7 layers of radial situated photosynthesizing parenchyma (two outer layers below epidermis contained a small cells, and inner layers parenchyma – greater cells). The cells of photosynthesizing parenchyma were closely joined. The central small vascular

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bundle with one vessel and small surroundings cells of phloem are situated centripetally below photosynthesizing parenchyma. The middle number of chloroplasts in parenchyma cell is equaled 7.7 ± 0.5 on the section. Very small air volumes were exposed only between cells of inner 3-6 parenchyma layers. Results of middle area tissues in submerged leaflet particles were next: epidermis – 12.9 %, photosynthesizing parenchyma – 83.7 %, conduction bundle – 2.29 % and vessel – 0.13 %. The middle area of small air cavities between inner layers of photosynthesizing parenchyma was amount 0.39 ± 0.03 %.

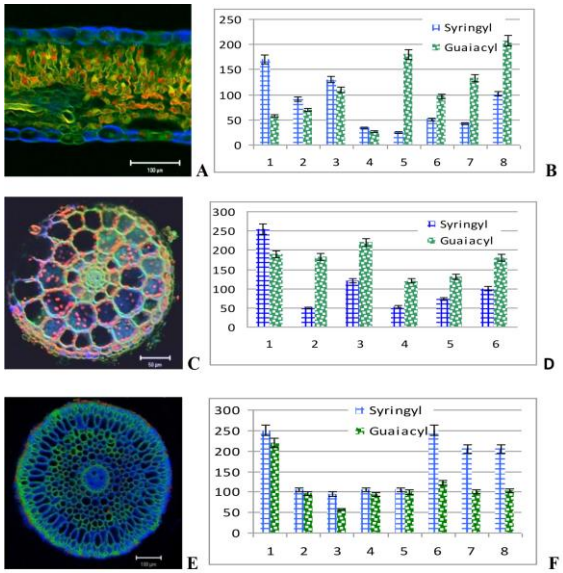

Fig. 2. Micrographs of cytochemical fluorescence of monolignols in the floating leaf (A) submerged leaf (C) and adventitious root (E) cells of *Trapa natans.* Localization of syringyl has blue fluorescence and guaiacyl—green fluorescence; chlorophyll—red auto fluorescence. Changes of the relative content (rel. units) of monolignols in cell walls of floating leaf (B), submerged leaf (D) and root (F). The key on figure B: adaxial epidemis (1, 2) abaxial epidermis (3, 4), 1, 3 – periclinal wall, 2, 4 – anticlinal wall, 5 – palisade parenchyma, 6 – spongy mesophyll, 7 – aerenchyma, 8 –vessels. The key on figure D: 1 – epidermis, periclinal wall, 2 – epidermis, anticlinal wall, 3 – photosynthesizing parenchyma, 4 – endoderm, 5 – phloem cells of conductive bundle, 6 – vessels. The key on figure F: 1- epidermis, 2 – exoderm, 3 – cortex, 4 – aerenchyma in cortex, 5 – endoderm, 6 – pericycle, 7 – stele phloem, 8 - vessels. Bars: A, E = 100 μm, C = 50 μm.

Adventitious roots. The shape of *T. natans* cross-section adventitious submerged roots is round; the middle size of root diameter is equalled 520 ± 23 μm. Roots revealed three distinct regions: outer cell region, cortical region and stele (Fig. 1E, F). Roots are covered by a monolayer epidermis that consists of small cells, by the middle size of 10 ± 2,1 x 7 ± 1,4 μm. Multilayer cortex is founded below the epidermis of root. The cortex was composed of parenchyma cells that are formed exoderm and endoderm. Outer layers of cortex contained large regions of radial aerenchyma (Fig. 1E, F), the amount of layers of that hesitates for different roots (from two to five). A cortex counts 8-10 layers of cells. The form of cortex cells from exoderm to stele is changed from oval to rounded or hexagonal. Chloroplasts were revealed in cells of root cortex (Fig. 2F), the middle number of chloroplasts in cell was 2.9 ± 0.8. The internal layer of cortex is formed thick-walled endoderm. Layer of pericycle is founded below endoderm. Almost rounded cells of pericycle have diameter 15 ± 1.7 μm, and characterizes by thick walls (Fig. 1F). Stele contains the little cells of phloem and four vessels with diameter about 20 ± 2.1 μm. Results of middle area tissues in submerged adventitious root were next: cortex with epidermis – 96.7 %, stele – 2.97 %, four vessels – 0.37 %. The middle area of air cavities (aerenchyma) between cortex cells was 18.9 ± 2.1 %.

Cytochemical analysis of monolignols in Trapa natans leaves and roots.

Flooding leaves. Cytochemical analysis of monolignols in the flooding leaves of *T. natans* are shown as blue fluorescence for syringyl and as green fluorescence for guaiacyl in the walls of epidermis, mesophyll parenchyma and in xylem vessels (Fig. 2A). The fluorescence intensity of DPKK-syringyl and DPKK-guaiacyl complex was different in the tissues (Fig. 2 B). Periclinal walls of epidermis (Fig. 2B; 3A, 3B) had the greatest fluorescence intensity of DPKK-syringyl complex in comparison with walls of mesophyll and conductive bundle cells, in which guaiacyl predominated. The size of S/G ratio in cells takes place in the next order: walls of epidermis > walls of spongy parenchyma

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and vessels > aerenchyma > palisade parenchyma. Intensity of monolignols (S+G) fluorescence in different cell walls was next: (in two epidermis) periclinal walls – 227 ± 19 and 240 ± 21; anticlinal walls –161±13 and 60 ± 9; palisade – 205 ± 19; spongy parenchyma – 148 ± 13; aerenchyma – 176 ± 19 and vessels – 309 ± 22 relative units.

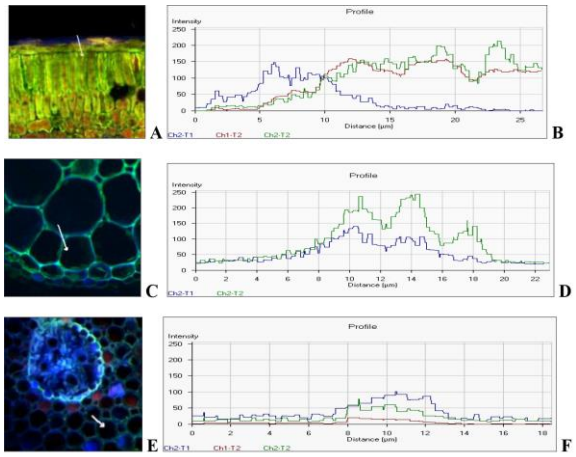

Fig. 3. Micrographs of cytochemical fluorescence of monolignols and histogram of fluorescence intensity of syrigyl, guaiacyl and chlorophyll auto fluorescence intensity in the floating leaf (A,B), submerged leaf (C,D) and adventitious root (E, F) cells of *Trapa natans.* On the histograms (B, D, F) of fluorescence intensity of syrigyl (blue line), guaiacyl (green line) and chlorophyll auto fluorescence intensity (red line) is shown. Ordinate —fluorescence intensity, relative units (pixels). Abscissa — distance (µm), which was scanned on the (A, C, E). This distance is shown as white line on the (A, C, E).

Submerged leaves. Like fluorescence of monolignols observed in submersed leaves. Cytochemical analysis of the complex DPKK-syringyl and DPKK-guaiacyl in submersed leaves shown blue fluorescence of syringyl and green fluorescence of guaiacyl in cell walls of epidermis, photosynthesizing parenchyma and central vascular bundle (Fig. 2C, 2D). There were some differences in fluorescence intensity of monolignols in cell walls of epidermis, in the photosynthesizing parenchyma and vessels. The level of fluorescence intensity of monolignols is presented in the Figure 2D, 3C and 3D (diagram). It is necessary to note that syringyl is predominated only periclinal wall of epidermis, whereas in walls of inner tissues guaiacyl is prevailed. On the size of the relation of S/G cells in submerged leaves are situated in such order: periclinal walls of epidermis > walls of endoderm > vessels and phloem conductive bundle > photosynthesizing parenchyma > anticlinal walls of epidermis. Intensity of monolignols (S+G) fluorescence in different cell walls was next: in epidermis periclinal walls – 444 ± 23; anticlinal walls 232 ± 20; photosynthesizing parenchyma – 340 ± 31; endoderm 173 ± 13; phloem of bundle – 203 ± 22 and vessels – 280 ± 23 relative units.

Adventitious roots. Cytochemical analysis of monolignols in *T. natans* adventitious roots is showed blue fluorescence of syringyl and green fluorescence of guaiacyl in the cell wall of epidermis, exoderm, cortex, aerenchyma, endoderm, pericycle, metaxylem, and vessels similar to that in submerged leaves (Fig. 2E). The level of luminescence intensity is presented on the figure 2F, 3E and 3F (diagram). The analysis of monolignols luminescence intensity showed that relative content of syringyl in walls of all tissues was more than that content of guaiacyl (Fig. 2F). The size of S/G ratio in cells takes place in the next order: pericycle and metaxylem > vessels > epidermis and aerenchyma > exoderm and endoderm walls. Intensity of monolignols (S+G) fluorescence in different cell walls of root was next: in epidermis – 470 ± 39; exoderm – 203 ± 24; cortex – 152 ± 13; aerenchyma in cortex – 198 ± 21; endoderm - 205 ± 20; pericycle – 373 ± 29; phloem in stele – 306 ± 31 and vessels – 308 ± 27 relative units.

Thus, anatomic study of *T. natans* submerged organs at the phase of plant flowering is showed forming of adventitious photosynthesizing roots that are formed from stem internodes near submerged dissected leaves, which some authors is named roots (Sculthorpe, 1967; Ishimaru et al., 1996). The previous structural investigation of vegetative organs of this species at the stage of vegetative growth did not revealed the presence of adventitious photosynthesizing roots on a submerged stem (Nedukha, 2013). We exposed that adventitious photosynthesizing roots began

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to appear at the beginning of flowering stage and they can to function far nuts formation. Like formation of adventitious photosynthesizing roots was early shown in aquatic plant *Cotula coronopifolia* and *Meionectes brownii.* (Rich et al., 2011; 2012).

Anatomic study of *T. natans* adventitious roots had shown the presence of well-developed aerenchyma that, as known, functions during of plant hypoxia for an accumulation and transport of gases. When hypoxia is arises in submerged organs (root, stem or leaves), then aerenchyma generate in such organs (Vartapetian, Jackson, 1997; Jackson, 2008; Jackson, Colmer, 2005). Besides, it is known that fundamental value of functioning of chloroplasts in providing of plant both the synthesis of carbohydrates and oxygen (Medvedev, 2004). Taking above noted data and the results of our investigation about the presence of chloroplasts in the cells of root cortex, we could to suppose that photosynthesizing cortex cells of *T. natans* adventitious roots with very developed aerenchyma participate actively in overcoming hypoxia that possible came at flowering stage. We suggest that oxygen, which store into cortex aerenchyma of adventitious roots, transport from aerenchyma cavities in both parenchyma cells and on the surface of root epidermal cells, where, as known, the oxidization of metals ions is occurred (to the reductive forms). Because, metal ions are able to settle on the surface of submerged adventitious roots, these ions block an absorption and transport of nutrients from a water environment into a root (Crawford, 1983).

An anatomic structure of needle-like particles of dissected submerged leaves of *T. natans* at the stage of flowering is differed greatly from structure of floating leaf. Centric structure, the presence of regular photosynthesizing tissue and chloroplasts in epidermis, the absent stomata and also poorly developed vessel system are promote to adaptation of such leaves to submerged environment by analogical to leaves of real hydrophytes or air-aquatic plants (Bercu, Fagaras, 2002; Bercu, 2004; Nedukha, 2011).

Besides, it is necessary to note that aquatic nut submerged dissected leaves don't have stele, exoderm and pericycle that is characteristic only for roots (Ezau, 1980). On the basis of the received our results and above marked information of literature, we consider the dissected green submerged organs of *T. natans* leaves, and pink adventitious roots as well as structure of auxiliary photosynthesizing roots, which is formed at the stage of plant flowering.

So, we showed the presence of syringyl and guaiacyl monolignols not only floating leaves, but also in submerged leaves and submerged adventitious roots of *T. natans*. The content of separate monolignols in investigated organs is depended on tissue type and plant organ. There were the general and differentia signs concerning these monolignols in leaves and roots. General signs were: 1) presence of syringyl and guaiacyl at the investigated species regardless of conditions of leaf growth; 2) almost identical (sufficiently great) content of S/G in epidermal walls and vessels cells of all organs, and 3) certain polarity of S/G ratio, that characteristic for every organ. Differentia cytochemical signs were: relative content of syringyl and guaiacyl in tissues of leaves and roots, and also different quantity of S/G tissues of all studied organs. Similar phenomenon was described in *Myriophyllum spicatum*, *Potamogeton pectinatus* and *P. perfoliatus* submerged leaves (Nedukha, 2015),

We revealed that nature submergence environment effected on increase of guaiacyl in photosynthesizing parenchyma of submerged leaf in comparison with that in palisade and spongy mesophyll cells of floating leaves. Besides we discovered the reliable increase of total amount monolignols (S+G) in epidermis and mesophyll of submerged leaves in comparison with that in floating leaves. Extremely high content of monolignols (S+G) was in both epidermis and in cell walls of root stele.

The revealed high content of syringyl, total amount monolignols (S+G) in cell walls of *T. natans* submerged leaves and adventitious roots can to explain the next manner. According to experimental data the increase

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of quantity of S/G, as a chemical barrier, is intensificated of cell defense from penetration of water and pathogens (Menden et al., 2007). Besides, the sign (S/G) testifies to the increase of mechanical durability of cells (Christiernin, 2006). Take account of these data and the evidence that the submerged leaves and submerged roots constantly are situated in a contact with a surrounding water micro flora, we suggest that the increase of monolignols in epidermis (notable in periclinal walls) serve as protection from possible invasion pathogens from submerged environment. Besides, it is possible, that more content of monolignols in walls of epidermal tissues of submerged organs also is helped opposition to water pressure on surface of submerged organs.

Conclusions

1. The study of an anatomic structure of *Trapa natans* adventitious roots shows well developed cortex, the presence radial aerenchyma and chloroplasts. Stele has a structure, which is typical for roots dicotyledonous.
2. The structure of *T. natans* submerged leaves differ from that of floating leaf. Mesophyll of the submerged leaves is not differentiated, its homogeneous with plenty of chloroplasts, stomata are absent.
3. Monolignols (syringyl and guaiacyl) were revealed in cell walls of *T. natans* submerged and floating leaves and also in adventitious roots by the laser scanning microscopy. The location and content of monolignols is depended from organs, tissue and environment conditions. It was found that natural flood is caused the distribution of monolignols on the tissues. The most content of monolignols was in cell walls of epidermis and vessels of submerged leaves and also in cell walls of stele and epidermis of adventitious roots.

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