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LIGNIN LOCALIZATION AND CONTENT IN *PHRAGMITES AUSTRALIS* IS SENSITIVE TO CHANGE IN WATER BALANCE ENVIRONMENT

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ABSTRACT
The sensitive of lignin localization and its content in leaves of *Phragmites australis* to change in the water balance of soil was established by using the cytochemical method, laser confocal microscopy, scanning electron microscopy and biochemical method. We investigated leaves of young plants *Ph. australis* (stage of vegetative growth), which used as a source of vitamins and to treat colds in medicine. The results were received by the comparison of the data in leaves of *Ph. australis* of the water-aquatic and terrestrial plants grown in nature. It was found that the decrease in soil moisture leads to an increase in the content of lignin in the epidermis and tissues of the conductive bundles of leaves of terrestrial plants. We assume that the increased of lignin content in the epidermis of leaves of terrestrial plants indicates that the leaves of both air-water and terrestrial plants of the reed can be used as a source of lignin for medical purposes.

KEYWORDS: Lignin, leaves, cytochemical and biochemical analysis, *Phragmites australis.*

INTRODUCTION
It is known that plant material serves humanity not only for food, but has long been used in medicine. This also applies to a lignin polymer containing phenolic hydroxyls groups characterized by an antioxidant property.[1, 2] Lignin is a biopolymer that has attracted the attention of many researchers because of its unique properties. Lignin 's degradability, biodegradability, high reactive potential, non-toxicity and cost-effectiveness of lignin has led to use it in various applications and industries. Lignin also use in the medical field due to its antibacterial and antioxidant properties, slow release of materials, reinforcement fillers, thermoplastic polymers and other biomaterials.[3-5]

Lignin is complex biopolymer of aromatic alcohols, which is synthesized in secondary cell walls. Lignin is highly branched and composed of cross-linked units, three monolignols: *p*-hydroxyphenyl, guaiacyl, and syringyl phenylpropanoid units.[6-8] Lignin synthesis is depending from tissue type, organ and species. 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 impassibility of water and solutions through walls. Besides, it was revealed that the change at tissue and cellular levels is accompanied by the increase of cellulose and lignin in plant grown under drought stress.[9,10] Researchers in many laboratories studies of participation of cell wall polysaccharides, including of lignin, in plant growth and development, especially in drought conditions.[11, 12] With regard to changes in lignin content in plants under influence of environment, but the data are opposite.[13-15] A decrease or increase in lignin content has been found in various experiments. It is possible; this depends on the plant species and growth phase used for the study. For example, a study of 50 miscanthropic genotypes showed the plasticity of the cell wall during drought, including changes in the content of cellulose, hemicelluloses and lignin, and found that changes in polysaccharide content were accompanied by a decrease in cell wall mass in stems and leaves.[10,16]

In nature, there are known plant species that can grow normally in water and in terrestrial soil. One of these species is *Phragmites australis* (Cav.) Trin. ex Steud, helophytes, which is characterized by a wide geographical distribution amplitude.[17,18] and by using in in agriculture, building industry and energy technologies.[19, 20] Vegetative organs (leaves, roots) and seeds of *Phragmites australis* is also used medicinally in the treatment of colds and bacterial-viral diseases.[21-23] This species grows both on the banks of rivers and lakes and far from water, in the field or in the mountains. Researchers found that the density and size of vascular bundles and the synthesis of sugars decreased and osmotic content changed in order to increase of water content in reeds growing in the mountains.[24] We suggest

that in the mechanism of tolerance of *Phragmites australis* plants growing in water and on drought soil is involved lignin, which can regulate the water transport by apoplast. Therefore, the aim of our work was to carry out a comparative analysis of the presence, distribution and content of lignin in the leaves of *Ph. australis* plants growing in water and soil with reduced moisture.

MATERIALS AND METHODS

Plant material and growth condition

Phragmites australis above-water leaves and leaves of terrestrial plants were used for study. Plants were harvested at the vegetative stage of plant growth on June 20-21, 2019. Air-water plants grew in water along-shore of the Venetian Strait (left Shore of Dnepr River, in Kiev, Ukraine) on the depth of 40-50 cm. Terrestrial plants grew near several 10-15 meters far from the shore in a sandy soil. The sun illumination (photosynthetic photon fluency rate (PPFR) on the adaxial surface of above-water leaves was 1470 μmol quantum.$m^{-2}\cdot s^{-1}$ and on the surface of leaves of terrestrial plants was equal to 870 μmol quantum.$m^{-2}\cdot s^{-1}$, accordingly. PPFR was measure by the means of the Light Meter LI-250 (USA, LI-COR). The temperature of water was + 24^{o}C and the temperature of air was + 27^{o} C. 12-13 plants of each ecotype were used for the biochemical investigations, for the microscopic investigations we used by three plants for each ecotype.

Laser confocal microscopy

The cytochemical method accordingly to Smith et al.[25] was used for the study of both distribution lignin and relative content of this biopolymer in walls. The live samples leaflets were stained for 7-10 min in 0.001% solution of auramine-O (sigma) dissolved in water, intensively washed with H_2O, then in 0.05 M phosphate buffer, post fixed in the solution of 1.0% paraformaldehyde in 0.05 M phosphate buffer, pH 7.2 at + 4^{o}C; then the samples were examined with laser scanning confocal microscope LSM5 (Zeiss, Germany). For detection of complex auramine-*O*-lignin fluorescence a laser was excited at 543 nm, the fluorescence emission detected at 598 nm wave length using an x 10, x 20 and x 40 objectives and the Pascal program. Chlorophyll auto fluorescence was excited at 440 nm and fluorescence emission detected at 660 nm. Three replications of cytological study of leaves were made.

Biochemical analysis

To determine the moisture content of the soil on which the terrestrial and air-water plants grew, the soil samples were taken at a depth of approximately 35-40 cm from the surface. Standard biochemical method was used to determine of the relative water content of the soil. This method based on drying the soil specimens in a thermostat at a temperature of 105°C to constant weight.[26] The soil humidity of air-aquatic plants was 79.3 ± 2.1%, and the soil humidity of terrestrial plants was 43.3 ± 1.7%. Content of lignin, was determined in 9-11 leaves using the standard protocols as described by Arasimovich and Ermakov.[26] The dry samples were used for the biochemical study. Three replications of each biochemical analysis were made. A value of biochemical results was expressed as the mean and standard errors. Statistical significance of relative content of lignin in cells was determined using a Studen's test ($P< 0.05$).

Scanning electron microscopy

The middle part of leaf blade from seven air-water and seven terrestrial plants were fixed in the solution of 3% paraformaldehyde and 1% glutaraldehyde (1 : 1, v) in 0.05 M phosphate buffer, (pH 7.4 for 3 h at 4^{o} C). Fixed samples were washed in the identical buffer, dehydrated in alcohol by the standard protocol.[27,28] Then the samples were critical point dried, mounted on aluminium stubs, coated with gold, and visualized in Japan scanning electron microscope (JSM 6060 LA at 30 kV).

RESULTS

Investigation of lignin in cell walls of leaf cross section.

Leaves of air-aquatic plants. Analysis of lignin on the sections of leaves of reed air-aquatic plants in the presence of a specific fluorescent indicator of auremine-*O* showed a bright yellow fluorescence in the cell walls of adaxial and abaxial epidermis, in vessels and bundle sheath of vascular bundles and in sclerenchyma (Fig. 1 a, b, c). Lignin fluorescence was virtually undetectable in mesophyll cells (Fig. 1 d, d''). The fluorescence intensity of the auremine-*O*-lignin complex was different in the tissues (Fig. 1 b', c', d'; Fig. 2, a). It was revealed that maximum frequency for lignin in the vascular bundles was 99601 (pixels, yellow line) at intensity and maximum the auto chlorophyll fluorescence was 803994 pixels, respectively (Fig. 1, i).

The content of lignin in the leaves of the air-aquatic plants was amount to 139 ± 6.1 mg/g DW.

Leaves of terrestrial plants. Similarly, cytochemical analysis of the complex auremine-*O*-lignin in leaves of *Phragmites australis* terrestrial plants showed yellow fluorescence of lignin in cell walls of epidermis and vascular bundles (Fig. 1, e, f, g, h). There were some differences in fluorescence intensity of lignin in cell walls of adaxial and abaxial epidermis, vessels and bundle sheath of vascular bundles. The level of fluorescence intensity of lignin is presented on the graph (Fig. 2 a) and histograms (Fig. 1 f', g' and h'). Luminescence intensity changes in leaves of terrestrial plants: 1.6 and 1, 3 times increased in cell walls of upper and lower epidermis, double – in walls of vessels, 1.3-1.5 – in walls of bundle sheath, and 1.2 – in sclerenchyma and bullifor cells. It was revealed that the maximum amount of fluorescence for lignin of leaves of terrestrial plants was double more (218821 pixels, yellow line) at intensity (Fig. 1, j), the maximum amount of auto fluorescence of chlorophyll in conductive bundles was

less (770814 pixels) than that in air-water plants (Fig. 1, j).

The content of lignin in the leaves of the terrestrial plants was amount to 155 ± 5.3 mg/g DW.

Investigation of lignin in cell walls of leaf epidermis surface.

Leaves of air-aquatic plants. To identify the types of epidermal cells, in which lignin is localized, early we examined the ultrastructure of leaf surface by a scanning electron microscopy. We gave a detailed description of the ultrastructure in our early work.[28] The study of the ultrastructure of the upper and lower surface of leaves *P. australis* growing in water showed that on adaxial and abaxial surface clearly distinguish two zones: the zone of stomata and the convex vault zone deprived of stomata (over veins) (Fig. 3, a, b).

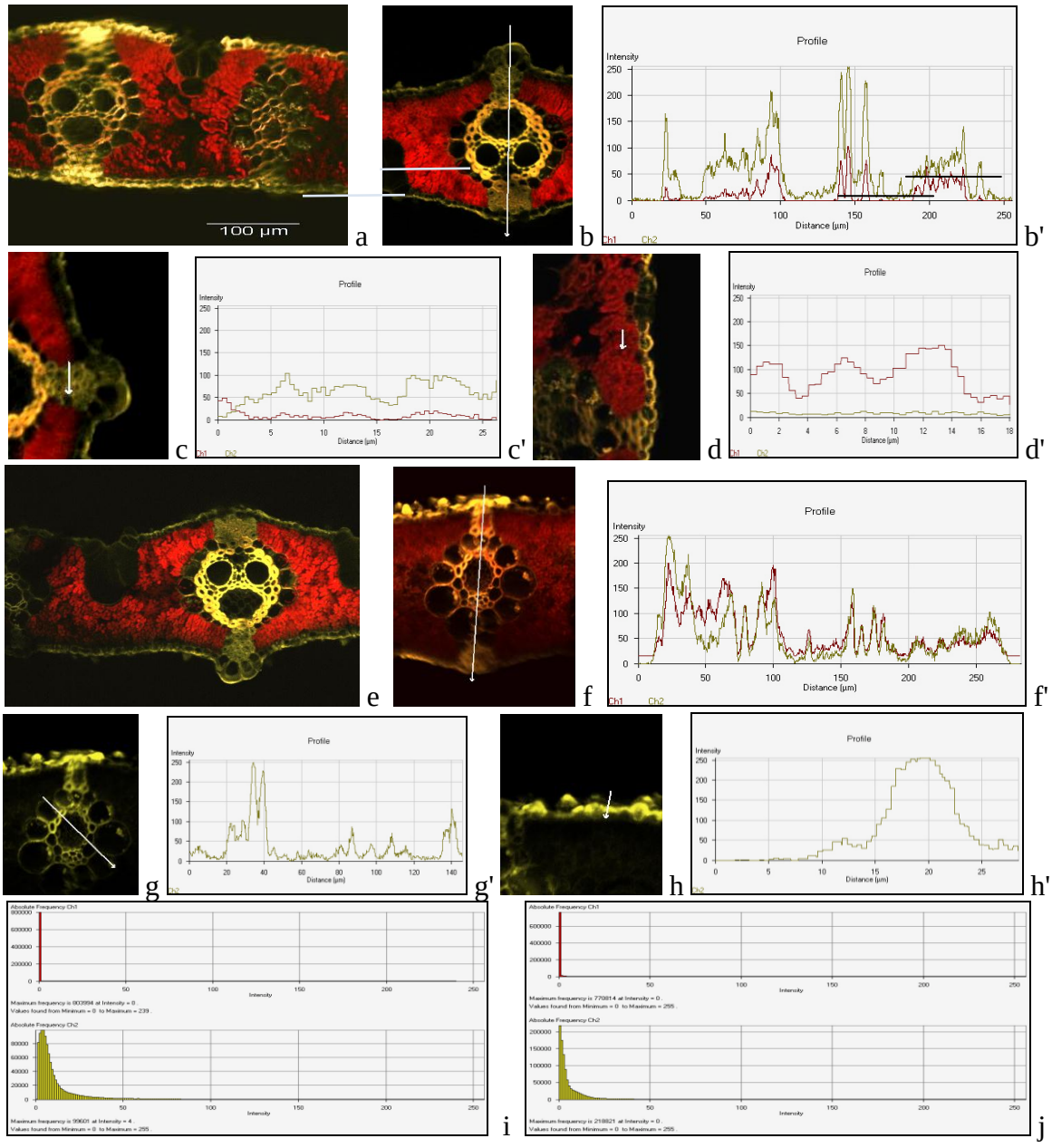

Figure 1: Micrographs of cytochemical fluorescence of lignin in leaf cells of *P. australis*. Cytochemical fluorescence of lignin in leaves of air-water (a-c) and terrestrial (e−h) reeds. Localization of lignin has yellow fluorescence, auto fluorescence of chlorophyll - red. Fig. b', c', d', f', g' and h' −histograms of fluorescence intensity of lignin (yellow line) and chlorophyll auto fluorescence intensity (red line). Ordinate −Fluorescence intensity, relative units; abscissa – Distance (mm), which was scanned on the figure b, c, d, g and h. This distance is shown as white line on the figure b, c, d, f, g and h. On figures i and j −absolute frequency of pixels for lignin (yellow graph) and for auto fluorescence of chlorophyll (red graph). Bars = 100 µm.

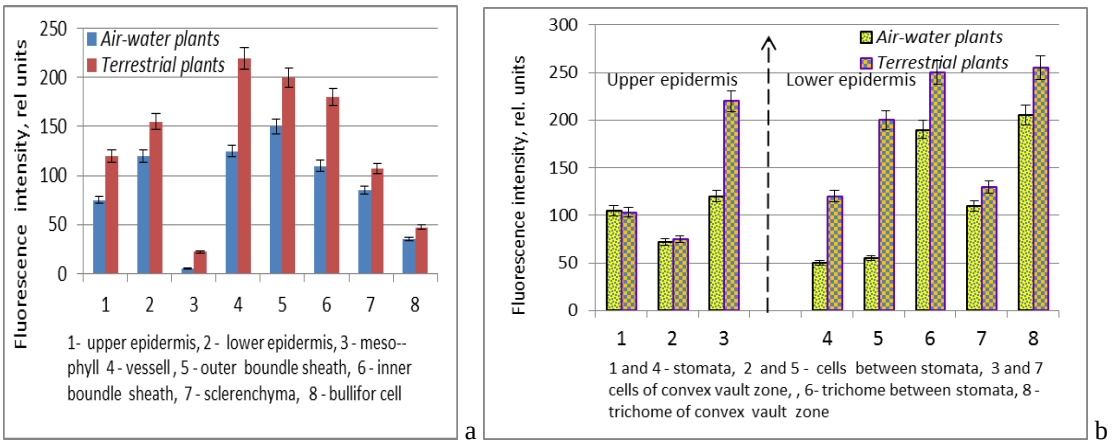

Figure 2: Fluorescence intensity of the auremine-*O*-lignin complexes in leaf cells of *Phragmites australis* air-water and terrestrial ecotype: a – in the cross sections of leaves, b – in cells of upper and lower surfaces of leaf blade.

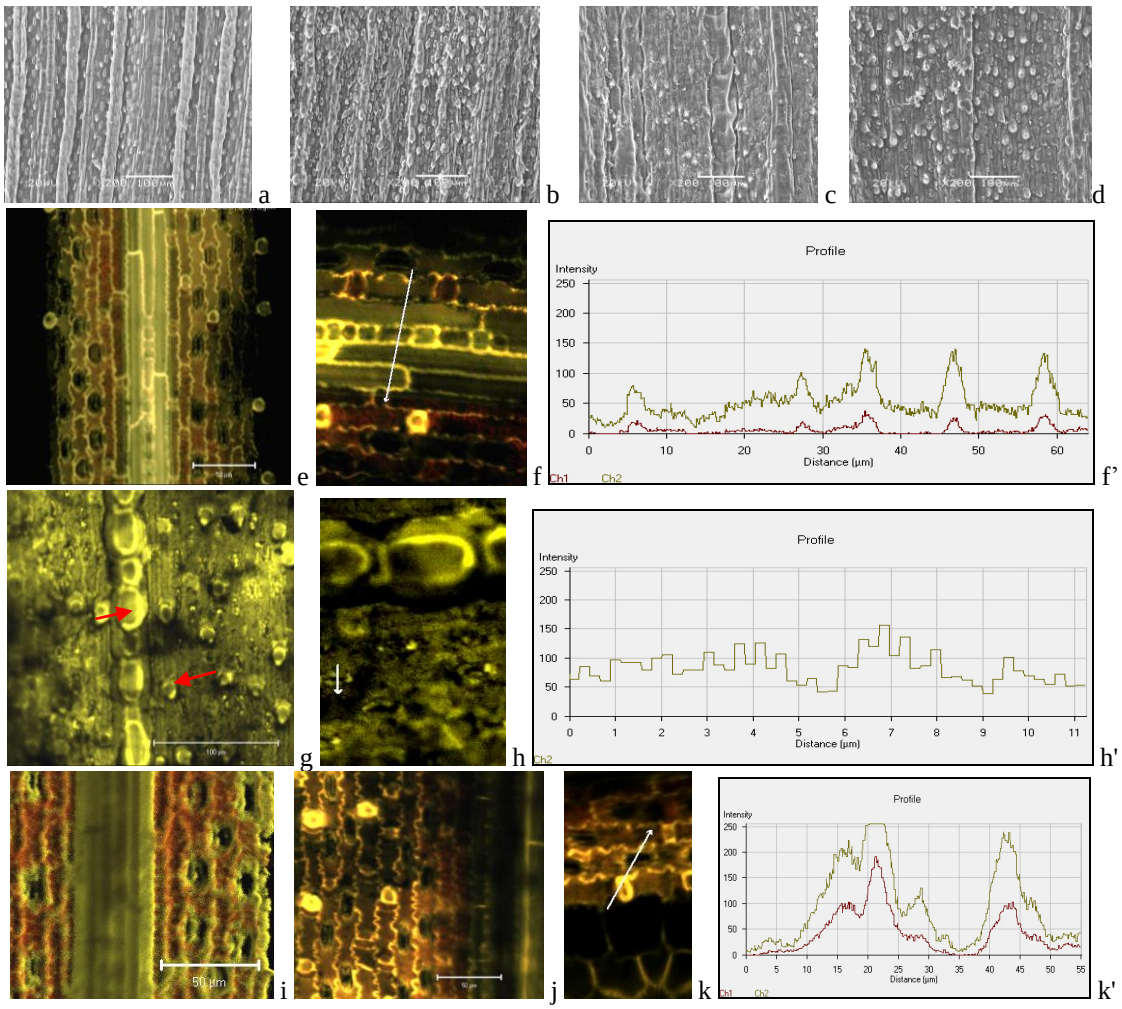

Figure 3: The structure of the adaxial (a, c) and abaxial surfaces (b, d) of leaves *Phragmites australis* air-water (a, b) and terrestrial (c, d) plants, the stage of vegetative growth. Micrographs of cytochemical fluorescence of lignin in cells of upper (e, f) and lower (g, h) surfaces of leaves of air-water (e-h) and terrestrial (i-k) plants. Localization of lignin has yellow fluorescence, auto fluorescence of chlorophyll – red. Fig. f', h' and k' – histograms of fluorescence intensity of lignin (yellow line) and chlorophyll auto fluorescence intensity (red line). Ordinate — Fluorescence intensity, relative units. Abscissa – Distance (µm), which was scanned on the figure f, h and k. This distance is shown as white line on the figure f, h and k. Bars: fig. a-d, g = 100 µm; fig. e, i and j = 50 µm.

The trichomes are situated in stomata zone. Cytochemical analysis of lignin in cells of upper (Fig. 3, e, f) and lower (Fig. 3, g, h) epidermis of leaves of air-aquatic plants of the reed showed a bright yellow fluorescence of the auremine-*O*-lignin complex in the periclinal and anticlinal cell walls of stomata and cells of convex vault zone over veins of upper epidermis and lower epidermis. The level of luminescence intensity is presented in Figure 2 b. The maximum amount of fluorescence for lignin in the adaxial epidermal cell was near 35241pixels, for abaxial epidermal cell – 37551 pixels, respectively.

Leaves of terrestrial plants. The study of the ultrastructure of the upper and lower surface (Fig. 3, c, d) of leaves *P. australis* growing on in a sandy soil showed that on adaxial and abaxial surface clearly distinguish two zones: the zone of stomata and the convex vault zone deprived of stomata (over veins). The trichome are revealed in stomata and convex vault zones. Cytochemical analysis of lignin on the upper (Fig. 3, i) and lower (Fig. 3, j) leaf surfaces of terrestrial cane plants showed yellow fluorescence of lignin in the cell walls of stomata and convex vault zone. The level of luminescence intensity is presented in Figure 2 b. The analysis of lignin luminescence intensity showed that relative content of lignin in walls of abaxial epidermis was more than that in cell walls of upper epidermis. The maximum amount of fluorescence for lignin in the adaxial epidermal cell was near 21332 pixels, for abaxial epidermal cell – 45731 pixels, respectively.

DISCUSSION

Thus, in the presented cytochemical research it has been shown that lignin is found in the cell walls of leaves of *P. australis* air-water and terrestrial plants in certain types' tissues. Lignin is observed in epidermis and vascular bundles, whereas it is very poorly in single mesophyll cells. However, the content of this polymer in epidermis and vascular bundles was reliable more in terrestrial ecotype leaves. Besides, the lignin shows the great intensity of fluorescence in cells of abaxial epidermis in comparison with that in adaxial epidermis cells regardless of ecotype plant. We observed lignin in cell walls as continuous layer, which recently were described in *Populus sieboldii* × *P. grandidentata* vessels,[29] sclerenchyma fibrils and sclereids of *Arundo donax* and *Phragmites karka* leaf,[30] in trichoma's and in epidermis internode of *Hordeum vulgare*.[31]

Lignin is the most important secondary metabolites synthesized by phenylalanine/tyrosine metabolism during the differentiation of distinct cell types in usual conditions and in response to environmental changes.[7,32] It is the second biopolymer that accounts for 30% of the organic carbon content of the biosphere and participates in the adaptation of the plant to change the water regime, altering the structure of the cell wall matrix, providing the passage of water and aqueous solutions through the cell walls of vessels.[7] It is known that many cell types can to synthesize lignin, including elements of conductive bundles, where lignin protects the hydro-mineral composition of cell juice from leaking and counteracts the effects of gravity in sclerenchyma and vascular tissue. Lignin also maintains the mechanical strength of the central axis of the organ from wind and rain, and participates in the mechanical anti-gravity support of vegetative organs and lignin is capable to reinforcement tensile strength of cell wall.[7,33]

The presence of lignin in the cells of bundle sheath of vascular bundle is like to that in differentiated tissues of xylem and phloem. As a complex phenolic polymer, lignin increases the cell wall stiffness of conductive bundles, increasing the hydrophobic plant walls, it is protects plants from invasion of various pathogenic fungi and microorganisms into leaf and stem cells.[34] The differences of the relative content of lignin in different epidermal cells which we revealed in *Ph. australis* leaves can be explained by the next. It is known that peroxidase and laccase enzymes are involved in lignin synthesis. These enzymes are key enzymes, involved in the polymerization of lignin monomers.[35,36]

Besides, plant protection during drought occurs not only at the cell level, but also at the molecular level. Researchers note increased enzyme activity of CoA reductase (cinnamoyl-CoA reductase, CCR and cinnamyl alcohol dehydrogenase and the expression of relevant genes.[13,37] Taking above noted and our results, we can to suppose that enhanced synthesis of lignin in the leaves of *Phragmites australis* terrestrial plants is due to activation of the respective enzymes. The phenomenon of an increase in the content of lignin in the leaves of the dry reeds, which grew under conditions of relative drought of the soil, is noteworthy. Extreme drought, like high salt stress, usually occurs at the same time and causes osmotic stress, which causes plant cells to lose water or even die, significantly inhibiting plant growth and development.[38,39] It is lignin that can decrease the flow of water by cells, which occurs with the complete or partial cessation of transpiration, which helps to support the osmotic balance of cells and cell integrity.[8]

The number of authors[8,22,40] reported that all vegetative and generative organs of *Eucalyptus* and *Phragmites australis* grown in tropics and subtropics are used in Chinese medicine, particularly in the treatment of diseases as bacterial meningitis, mouse hepatoma, melanoma, an arthritis, bronchitis, cancer, cholera, cough, diabetes and many other diseases. In addition to lignin and phenols, this species of plant also contains large amounts of amorphous and crystalline silicon in the leaves, as we recently reported.[28] Silicon is known to be a unique chemical element that reflects ultraviolet light,[41] making it possible to also use *Fragmites australis* leaves in the development of special cosmetic sun care products and sunglasses by analogy with other plant species.[4] Based on the results of our experimental investigations and data of above-noted literature, the next

conclusions could be made; leaves of *Ph. australis* in vegetative stage, which grows both on the banks of rivers and lakes, and on the dry land of Ukraine, are a convenient material for use in medicine and pharmacology.

Thus, we found that regardless of the conditions of plant growth, reed leaves in the vegetative phase of plant growth contain a fairly large amount of lignin (up to 10% in dry weight and 30% - in recalculation for fresh weight). Taking into account the fact that the leaves of the queue, which grows both on the banks of rivers and lakes, and on the dry land, are a convenient material for use in medicine and pharmacology.

CONCLUSION

The cytochemical, laser confocal microscopic and biochemical study revealed that content and distribution of lignin in epidermis and conductive bundles of leaves in *Phragmites australis* in vegetative stage growth dependent from soil moisture on which this species grown.

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