TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Research Article

Turk J Bot
(2022) 46: 459-472
© TÜBİTAK
doi:10.55730/1300-008X.2722

Effects of moderate drought on leaf bulliform cells of aquatic and coastal population of *Phragmites australis*

Olena M. NEDUKHA*
Department of Cell Biology and Anatomy, Institute of Botany, National Academy of Sciences of Ukraine, Tereshchenkivska Str. 2, Kyiv, Ukraine

Received: 05.03.2022 • **Accepted/Published Online:** 03.08.2022 • **Final Version:** 19.09.2022

Abstract: Studying leaves of aquatic and terrestrial populations of *Phragmites australis* grown on the bank of the Venetian strait of the Dnipro River in Kyiv (Ukraine) for establishing the role of bulliform cells in the mechanisms of plant resistance to moderate soil drought were studied. Bulliform (motor) cells participate in the twisting and folding of leaves during drought and strong sunlight. The study of bulliform cells in the leaves of the aquatic and terrestrial populations of *P. australis* was carried out using the methods of light microscopy, cytochemical methods, laser confocal microscopy, and biochemical methods. The comparative analysis of the structure of bulliform cells of leaves of *P. australis* has shown significant differences depending on plant growth location. The differences in the number, size, and area of bulliform cells and also polysaccharide content showed clear phenotypical plasticity. Cytochemical and laser confocal microscopic studies of polysaccharides of cell walls in bulliform cells of aquatic and terrestrial ecotypes of *P. australis* showed that a decrease in soil moisture in a natural moderate drought of soil led to an increase in lignin and syringyl monolignol content in the outer walls of bulliform cells and also to a decrease in cellulose and callose content in outer and inner cell walls. The obtained data shows that the studied signs of bulliform cells can be markers of tolerance for population plants that have the ability to curl leaves for the preservation of optimal water balance in moderate drought.

Key words: Motor cells, reed leaf, cell wall, wall's polysaccharides, monolignols ratio, laser confocal microscopy

1. Introduction

Drought has been called the most dangerous climate hazard facing our planet's population (Kogan, 1997). One of the key challenges for plant science is to improve our understanding of how drought affects plant ecology and plant functional traits, as this will impact agricultural productivity as well as vegetation management (Marchin et al., 2020). During soil drought or extreme temperature rise, the plant activates all possible structural and functional systems of metabolism for survival in unfavorable conditions. A decrease in respiratory and cuticle transpiration, thickening of cell walls, and an increase in deposits of lignin and wax in the cell walls of leaves occur during drought (Kerstein, 1996). Grigore et al. (2010) reported that the decrease in transpiration can also occur by reducing the area of water evaporation and the area of absorption of light by leaves by rolling or folding them. Leaves of many species are known to be either folded or rolled into tubes during drought (Grigore et al., 2010). One of the most promising, natural wetland bioenergy crops is common reed, *Phragmites australis* [Cav.] Trin. ex Steud, an intensely studied, tall grass species of the Poaceae family. Reed is a cosmopolitan species with a near-global distribution and great socioeconomic importance. The high intraspecific diversity of this species makes it ideal for studying acclimation and adaptation processes, as well as responses to global climate change (Brix, 1999; Mahmoud Elhaak et al., 2015; Hong et al., 2019), when soils are polluted with heavy metals (Eller et al., 2020), and when exposed to biotic factors (pathogens) (Ricardo et al., 2021). *Phragmites australis* has been observed throughout most continents, such as Africa, America, Asia, and Europe (Köbbing et al., 2013). This species' morphology and/or population structure may have an impact on ecosystems (Park et al., 2018). Studies on the differences in morphology and/or population structure are of importance in understanding the ecological role of this species, particularly in various habitat types (Hong et al., 2019).

It was established that twisting the leaf while exposed to sunlight and heat can reduce the surface of the leaf turned to the solar stream. It is believed that the process of mechanical folding or twisting of the leaf plate occurs with the participation of bulliform cells, which are also called

* Correspondence: o.nedukha@hotmail.com

motor cells (Esau, 1969; Grigore et al., 2010). Bulliform cells are located in groups next to the usual cells of the epidermis, forming a single layer; these cells are filled with water, and these cells have no chloroplasts. There are several points of view regarding the function of bulliform (motor) cells: i) motor cells involved in the development of young leaf blades (Grigore and Toma, 2011; 2014); ii) these cells participate in the hygroscopic twisting of already developed leaves during air or soil drought, altering leaf cell turgor and decreasing leaf transpiration, respiration, and overheating (Dickison, 2000).

Adaxial (outward) and abaxial (inward) leaf rolling have been described (Candela et al., 2008). Leaf rolling was shown to be one of various defense strategies against adverse environmental conditions. The concentration of polyamines, phenolic acid and low-molecular-weight carbohydrates increases during leaf twisting as a reaction to water deficit stress. In addition, brassicasterols and cytokinins found in leaves have a strong activity (Kadioglu and Terzi, 2003). When leaves are twisted, motor cells lose turgor and shrink; when these cells are saturated with water again, the leaves straighten up (Price et al., 1977). When halophytes and desert plants were compared to mesophyte ecotypes, bulliform cells were found to be more developed (Weber et al., 1977). Now, genes have been identified that are involved in the phenomenon of leaf rolling. In particular, in the rice leaf, the *ACL1* gene (Abaxial Curling Leaf 1) has been identified, which is expressed both during leaf twisting and during the formation of a particular leaf shape (Li et al., 2010). It remains unclear whether the cell walls of bulliform cells respond to external influences by changing their structure and polysaccharide content. The following hypothesis is proposed in order to address the bulliform cells' mode of action:

i) The stiffness and softening of motile cell walls may be affected by the monolignol ratio.

ii) Water can pass through the apoplast due to callose deposition in the motor cell walls.

iii) The altered responses to soil moisture may be due to differences in the polysaccharide and monolignol content of motor cell walls.

2. Materials and methods

2.1. Plant material and growth condition

In June, *Phragmites australis* (Cav.) Trin. ex Steud (Poaceae) leaves were collected from aquatic plants growing in shallow water of the Venetian Strait of the Dnipro River (Kiev, Ukraine) and terrestrial reed plants growing in coastal zone with sand soil, near 10–15 m away from the shore (Figures 1a and 1b). The selected plants were in a vegetative growth stage. The water temperature on the days of plant fixation ranged from 23 °C to 24 °C and the air temperature ranged from 25 °C to 27 °C. The solar illumination (photosynthetic photon fluency rate, PPFR) on the adaxial surface of the leaves of air-water plants ranged from 1230 to 1400 μmol quantum $m^{-2\ s-1}$, and the illumination of leaves of terrestrial plants ranged from 670 to 810 μmol quantum $m^{-2\ s-1}$, accordingly, on the days of collection of plant material. The PPFR was determined using the Light Meter LI-250 (USA, LI-COR).

To determine the moisture content of the soil on which the terrestrial and aquatic plants grew, the soil samples were taken at a depth of approximately 35–40 cm from the surface. The standard method was used to measure the water content in leaves and in soil. This method is based on drying soil specimens to a constant weight in a thermostat at 105 °C (for soil) and 85 °C (for leaves) (Ermakov, 1982). The soil humidity on which aquatic plants grew was 79.9 ± 2.1%, and the soil humidity, on which terrestrial plants grew, was 43 ± 3.3%.

2.2. Light microscopy

The segments of leaflet of aquatic and terrestrial reed plants grown in water and terrestrial soil on shore zone were fixed in the solution consisting of 2% paraformaldehyde and 1% glutaraldehyde (in ratio of 1:1, vol) in 0.5 M phosphate buffer, pH 7.2 over 24 h. They were then rinsed three times in 0.5 M phosphate buffer, pH7.2; and were dehydrated in a graded series of ethanol dilution (30%, 50%, 70%, 90%, and 100% ×3, v/v) and acetone and subsequently embedded in an epon-araldite resin mixture (epoxy embedding medium — 30%, epoxy embedding medium hardener — 40%, araldite M — 20% and epoxy embedding medium acceleration — 10%). The samples were kept for 24 h at 20 °C, and then transferred to a thermostat for 12 h — at 37 °C and 24 h — at 56 °C until the complete polymerization of the resin according to the protocol of Weakley (1975). For light microscopic observation, the sample sections were stained with 0.1% w/v Safranin *O* for 12 h, washed and stained with secondary dye (0.05% Fast Green) according to the protocol (Pausheva, 1988). The samples were visualized using a NF light microscope (Carl Zeiss, Germany). The seven samples of aquatic plants and seven samples of terrestrial plants were used to measure the density of bulliform cells in leaves.

2.3. Biochemical analysis

The lignin content was determined by the standard method (Jenson, 1965), which is based on the extraction of lignin from tissue with sodium chlorite in the presence of acetic acid. For one experiment, we used 5 g of samples (fresh samples). The ground material was filled with an aqueous solution containing 160 mL of water, 1 mL of acetic acid and 1.5 g of sodium chlorite ($NaClO_2$) for 2 h, then (every hour) was added 50 mL of freshly made sodium chlorite solution. After 5 h of lignin extraction, the residue of the material from which the lignin was removed was thoroughly washed with water on a special filter and

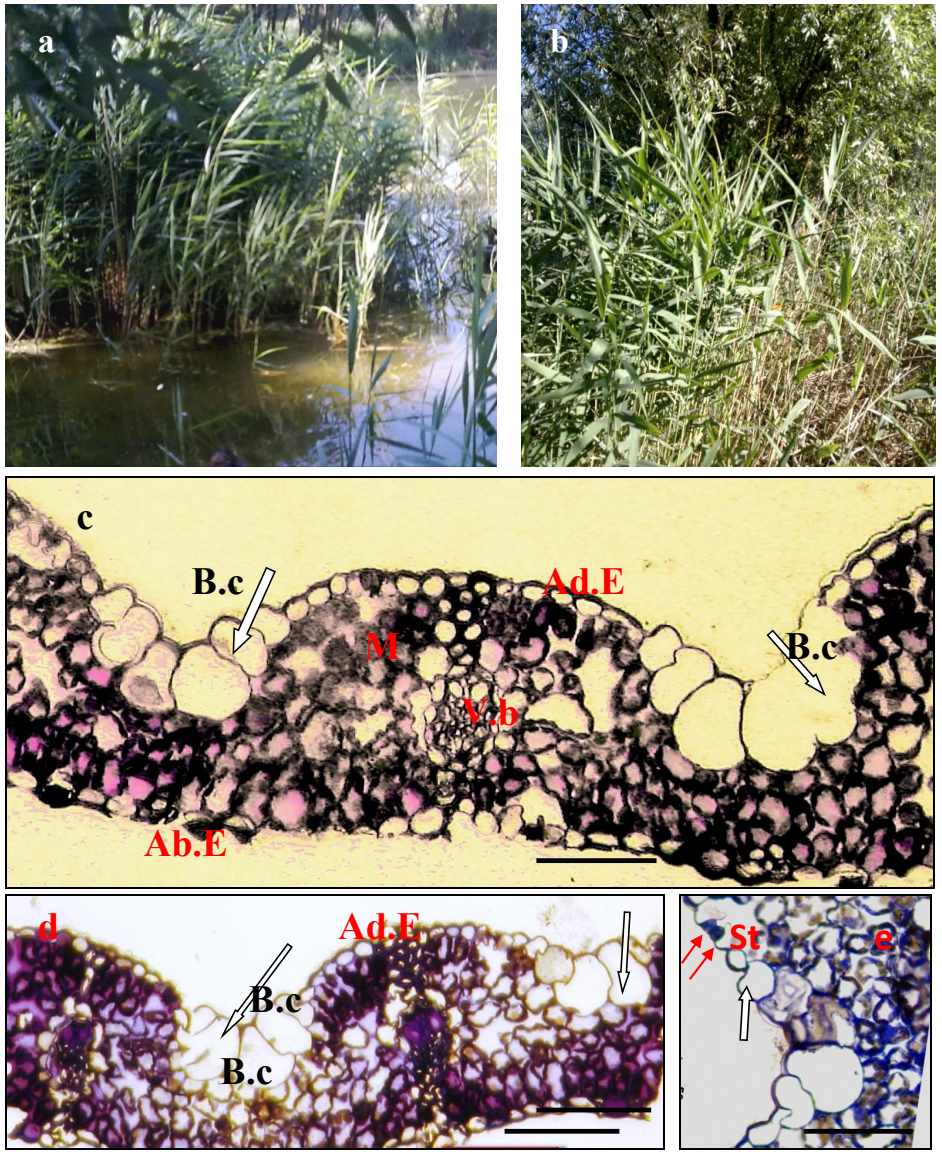

Figure 1. *Phragmites australis* aquatic (**a**) and terrestrial (**b**) plants. Light microscopic micrographs of cross sections of leaf blade of aquatic (**c**) and terrestrial (**d, e**) reed plants. Abbreviation: **Ad.E**: adaxial epidermis, **Ab.E**: abaxial epidermis, **B.c**: bulliform (motor) cell, **M**: mesophyll, **St**: stomata, **V.b**: vessel bundle. The white arrows indicate the bulliform cells, red arrows – stomata. Scale bars: **c, d, e** = 200 µm.

dried in a thermostat at 85 °C to constant weight, then the dry residue was converted to fresh weight, the difference between after isolation of lignin, the content was calculated and converted to fresh and dry weight, respectively. The experiments were repeated three times.

The cellulose, hemicellulose and pectin content were determined by the method according to Arasimovich and Ermakov (1987). The method is based on the initial extraction from sugars from 5 g of fresh samples by treatment with 82% ethyl alcohol, then the gradual extraction of hemicelluloses with 5% and 25% potassium hydroxide (KOH) solutions, followed by hydrolysis of amorphous cellulose with 12% hydrochloric acid (HCl) solution and crystalline cellulose with 72% sulfuric acid (H_2SO_4) solution. Five grams of samples (fresh sample, central part of the leaf blade) in one experiment was used.

Isolation of pectin from leaf samples was realized according to the protocol of Arasimovich and Ermakov (1987). To isolate water-soluble pectin from the samples weighing 20 g was carried in the following way: first, the sugars were removing with 80% alcohol, and then the rest of the material was filled with hot water at 45

°C for 30 min, stirring on a magnetic stirrer. Extraction of soluble pectin with hot water is repeated three times. After removing the soluble pectin, the residue of the plant material is first treated with 0.3n hydrochloric acid and heated for 30 min in a water bath, then the solution is filtered and washed three more times with hot water (up to 50 °C). The filter with the remainder of the material was transferred to a flask and filled with 1% tri-ammonium citrate ($C_6H_{17}N_3O_7$) solution and left at 90 °C for 30 min. The extract was filtered and washed with hot water and, after cooling, adjusted to the mark. The resulting extract contained water-insoluble pectin. The experiments were repeated three times.

2.4. Cytochemical analysis, laser confocal microscopy

Cytochemical study of the localization and distribution of cellulose in the cell walls of reed bulliform cells was performed according to the W. Herth (1980) protocol. Leaf samples prefixed in 1.5% paraformaldehyde were washed thoroughly with phosphate buffer and then incubated in 0.001% aqueous solution of calcofluor White (Sigma) in phosphate buffer (pH 7.2) for 15 min, washed with the buffer. The fluorescence analysis of cellulose was performed using a confocal laser microscope. The calcofluor-cellulose complex gives blue fluorescence at an excitation wavelength of 440 nm and an emission wavelength of 505 nm.

Localization and relative content of callose in the cell walls were determined using the classic method by H. Currier (Currier and Shin, 1968). The samples were prefixed in 1.5% paraformaldehyde solution in 0.05 M phosphate buffer, pH 7.2, stained with 0.05% solution of aniline blue 0.5 M K_2HPO_4 for 15–20 min at 20 °C, washed thoroughly with water and immediately were examined in a confocal laser microscope. The callose-aniline blue complex had a green fluorescence at an excitation wavelength of 380 nm and an emission wavelength of 505 nm.

To determine the localization and relative content of lignin, the living material was incubated in 0.001% aqueous auramine-*O* solution for 5–10 min according to the protocol (Smith et al., 1986). The fluorescence analysis of lignin was performed using a confocal laser microscope. The detection of lignin was performed at an excitation wavelength of 543 nm and an emission wavelength of 598 nm. The auramine-lignin complex had a yellow fluorescence. Study on the localization and relative content of monolignols i.e. syringyl and guajacyl was performed by the cytochemical method according to the protocol (Wuyts et al., 2005; Djikanovic et al., 2007). Live leaf samples were incubated in 0.25% 2-aminoethyl ester of diphenyl carboxylic acid (DPBA) for 20 min, washed thoroughly with water, and fixed in 1.5% paraformaldehyde solution on phosphate buffer. The fluorescence analysis of syringyl and guajacyl was performed using a confocal laser microscope. Syringyl detection was excited at wavelength of 340 nm and an emission wavelength of 430 nm. The DPBA-guajacyl complex was green, respectively, guajcyl detection was excited at 450 nm, the fluorescence emission detected –520 nm wavelengths using X10, X20 and X40 objectives. Chlorophyll autofluorescence was excited at 440 nm and fluorescent emission was detected at 662 nm.

2.5. Statistical analysis

The experiments were performed in a randomized design with three replications. Statistical differences between mean values were determined by the Student's t-test. Differences at the level of $p < 0.05$ were considered as significant. Each time, three to four leaves were excited from three aquatic plants and from three terrestrial plants to obtain the anatomical parameters of buliform cells and leaf blade, the cytochemical parameters of cellulose, callose, lignin and monolignols, and also biochemical parameters. Analysis of variance for all measured variables was performed by Origin 6.1 programs. The fluorescence intensity of polysaccharides was performed by Pascal program (LSM 5).

3. Results

3.1. Anatomical features of *Phragmites australis* leaf bulliform cells

The anatomical structure of the leaves of aquatic and terrestrial *Phragmites australis* was studied for the identifying of places of bulliform cells and for the study of wall polysaccharides and lignin localization in bulliform cell. The leaves of *P. australis* plants, independent of location, were of the amphistomatic type and characterized by a linear shape (Figures 1a and 1b). Parallel convex veins were clearly visible on the adaxial surface. The leaves were narrow and long (Table 1), and they slightly twisted towards the adaxial surface. The water content in the leaves of aquatic plants was 62.8 ± 0.5% and in the leaves of terrestrial plants it was 57.2 ± 0.7%, respectively.

The next signs were established for leaves of *P. australis* aquatic plants grown in the Venetian Strait: the thickness of the leaf blade on the transverse section was not uniform, in the area of the surface valley, it varied from 200 ± 15 to 380 ± 17 μm, the area of the surface peak, where the vessel bundles were placed, the thickness section varied from 300 ± 13 to 600 ± 31 μm. A study of the crosssections of the leaf blade showed that the leaf structure was isolateral. Bullifor (motor) cells were situated on the adaxial surface between two vascular bundle ridges in parallel with the veins, and these cells were bordered by the usual epidermal cells. Bulliform cells are groups of large transparent cells (Figure 1c), which are closely adjacent to both mesophyll cells and the epidermis. The cell walls of bulliform cells were very thin. Motor cells were typically arranged in groups of 6–7 cells, and the size of central cells was larger than lateral

Table 1. The measured parameters of *Phragmites australis* plants grown in water and terrestrial soil. An asterisk denotes significant differences in the parameters in aquatic plants from terrestrial plants (means ± SE).

The measured parameters	The data of measured parameters of *P. australis* grown	
	In water	In terrestrial soil
Moisture of soil, %	79.3 ± 2.70	47.0 ± 2.30**
Moisture of leaves, %	62.8 ± 0.5	57.2 ± 0.70*
Size of central bulliform cell, μm:	140 ± 15	100 ± 11*
long axis short axis (at the inner pole, adjacent to the mesophyll)	88 ± 9.1	70 ± 8.0*
The average area of one group of motor cells, μm^2	5701 ± 310	4844 ± 297*
The average area of the mesophyll, which accounts for one group of motor cells, μm^2	28870 ± 1410	36607 ± 1770**
The ratio of the area of motor cells to the area of mesophyll cells (between two vessel bundles)	1:5.26	1:7.56

(* $p \leq 0.05$; ** $p \leq 0.01$).

cells. The arrangement of motor cells in the architecture resembles a semicircular arc; the upper part of these cells in the section forms a fan-shaped structure. The long axis of the central bulliform cell averaged 140 ± 15 μm; and the short axis (at the inner pole, adjacent to the mesophyll) – 88 ± 9.1 μm. The long axis of lateral cells was 67 ± 9 in average and the short axis – 41 ± 3 μm, accordingly. The average area of one group of motor cells was 5.26 times smaller than the area of the mesophyll and it was presented in Table 1.

Leaves of terrestrial reed plants in morphology and anatomy were similar to those of aquatic plants. Clearly visible elevated conductive bundles of vessels parallel to the long axis of the leaf blade were shown on the adaxial surface (Figure 1d). The thickness of the leaf blade on the transverse section was not uniform. In the area of the surface valley, it varied from 150 ± 13 to 230 ± 19 μm, in the zone of the surface peak, it ranged from 250 ± 12 to 330 ± 27 μm. Bulliform cells were situated between vascular bundles, which were similar to those in the leaves of aquatic plants. The bulliform cells were grouped by 4–5 cells, which were also located in depressions. The inner walls of marginal buliform cells are contacted with stomata cavity (Figure 1e). The outer parts of the motor cells were narrowed near the epidermis cells and expanded in the area of contact with the mesophyll. The architecture's arrangement of bulliform cells is similar to that of reed leaves growing in water and also resembles a curved arch. The upper part of motor cells in the section formed a fan-shaped structure. The long axis of the central bulliform cell measures 100 ± 11 μm on average, and the short axis (at the inner pole, adjacent to the mesophyll) measures 70 ± 8 μm. The long axis of lateral cells was less; it was an average of 51 ± 7, and the short axis –20 ± 3 μm, accordingly. The average area of the group of bulliform cells in the leaf of the terrestrial reed ecotype was 7.56 times smaller than the area of the mesophyll, and it is presented in Table 1.

3.2. Cytochemical study of cellulose, callose, lignin and monolignols in bulliform cells.

The cytochemical study of wall polysaccharides, including of cellulose, callose, and lignin in bulliform cells of *P. australis*, grown in water and on terrestrial soil, has shown an analogous location of the studied polysaccharides in the inner and outer walls of bulliform cells.

The study of the localization of cellulose in the presence of the specific fluorescent indicator calcofluor revealed a bright blue color in the bulliform cell walls of a leaf in *P. australis* grown in water (Figures 2a and 2b). The intensity profile along the calcofluor-cellulose complex was different in the periclinal (outer) and inner walls (Figures 2b and 2c). The highest intensity profile of cellulose was found in the outer walls of bulliform cells. It was 190 ± 11 relative units of fluorescence; in the inner walls of motor cells, its intensity was less and was 154 ± 12 relative units. The cytochemical study of cellulose in bulliform cells of terrestrial plants has shown an analogous location of the studied polysaccharide (Figure 2d). But the intensity of cellulose fluorescence differed from that of aquatic plants. The intensity profile of the calcofluor-cellulose complex in the outer periclinal walls of bulliform cells was twice as high as that in the inner walls, and it was 170 ± 11 and 78 ± 5.9 relative units, accordingly.

Callose in the presence of the specific fluorescent indicator aniline blue was green in the bulliform cell walls of *P. australis* grown in water (Figures 2e and 2f). The intensity profile in the image along the aniline blue-callose complex was different in the periclinal (outer) and the inner walls; in the inner walls its value was twice less than in the outer cell walls (Figures 2f and 2g). Callose was also in green color in the bulliform cell walls of *P. australis*

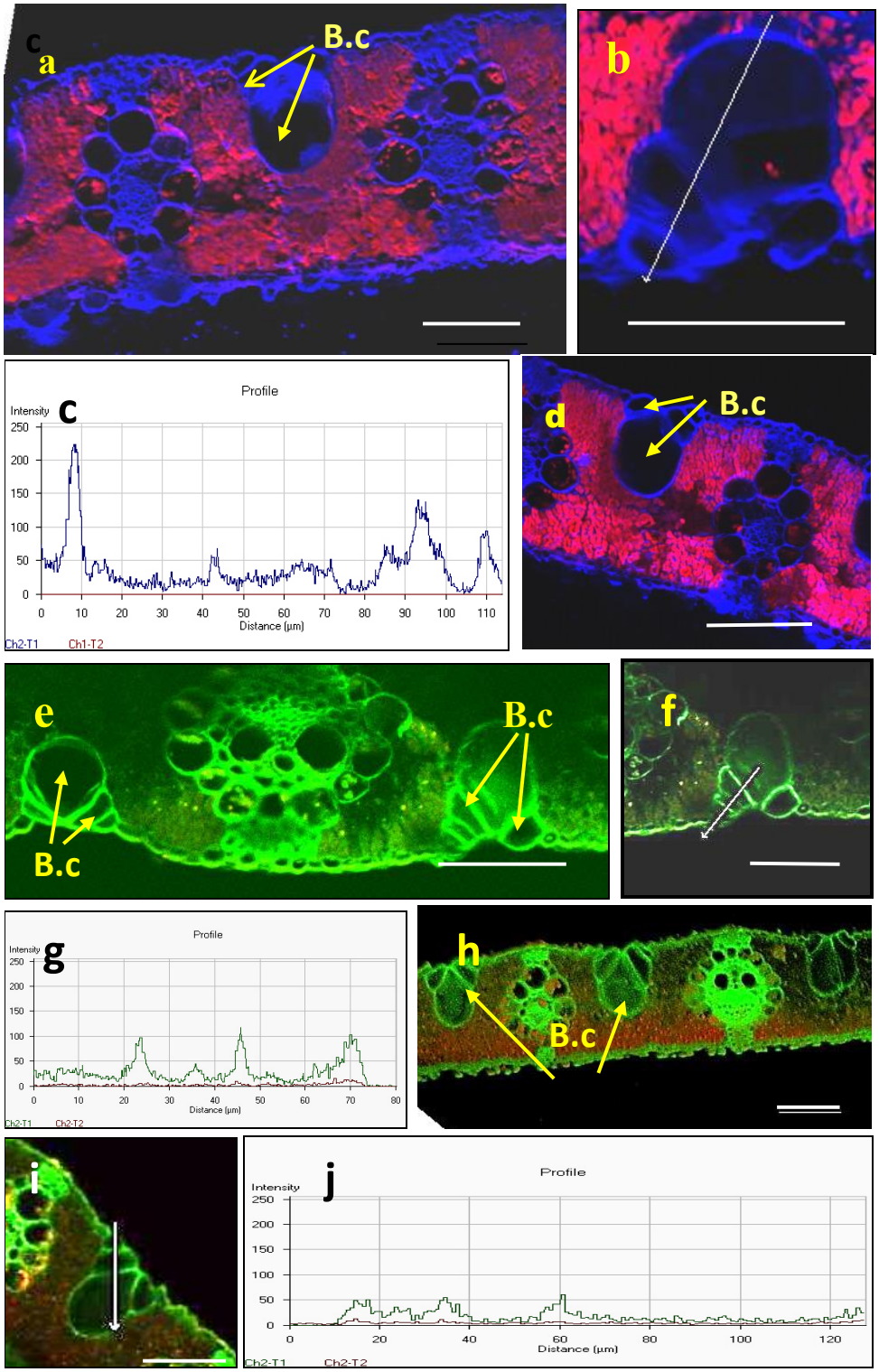

Figure 2. Confocal laser scanning microscopic images of fluorescence cellulose (**a-d**) and callose (**e-i**) in walls of the bulliform cells in leaves of *Phragmites australis*, grown in water (**a, b, e, f**) and on terrestrial soil (**d, h, i**). The leaf sections treated with 0.001% calcofluor white solution (marker of cellulose) or 0.05 M aniline blue (marker callose) for 15 min in the dark. Cellulose has blue fluorescence, callose — green. In the figure **c** — histogram of intensity profile of cellulose (blue line), in the figures **g** and **j** — histogram of intensity profile of callose (green line). Ordinate — intensity of profile, relative units; abscissa — distance was scanned on the **b, f** and **i**; this distance is indicated as white arrow on the **b, f** and **i.** Scale bars: 100 µm.

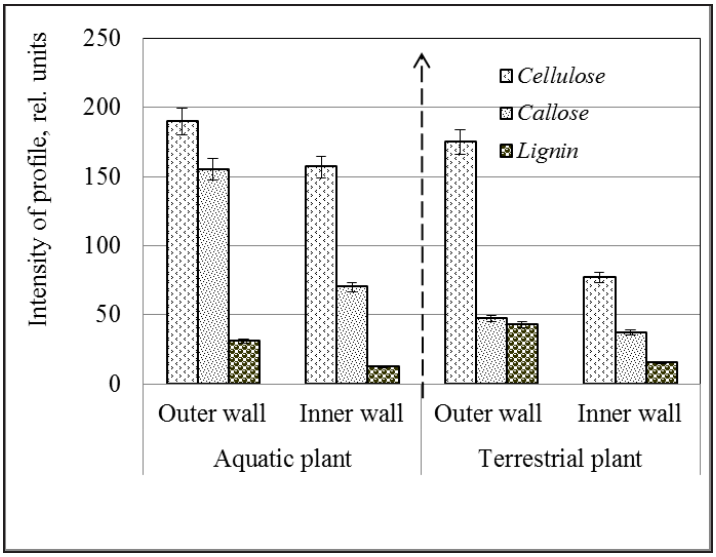

Figure 3. The intensity profile of cellulose, callose, and lignin in the walls of leaf bulliform cells of *Phragmites australis* grown in water and on terrestrial soil. Data was obtained using the Pascal program and laser confocal microscopy. Statistical significance of relative content (or intensity of profile) of cellulose, callose and lignin in the cell walls were determined using Origin 6.1, including the standard error (means ± SE).

grown in terrestrial soil (Figures 2h and 2i). The intensity profile of the image along the aniline blue-callose complex in the outer walls of bulliform cells was higher than in the inner walls (Figures 2i, 2j, and 3).

Lignin in the presence of the specific fluorescent indicator auremine-*O* has a bright yellow fluorescence in the bulliform cell walls of *P. australis* grown in water (Figures 4a and 4b). The intensity profile of the auremine-lignin complex in the walls was very little in comparison with that of cellulose and callose fluorescence in the walls of the bulliform cells (Figures 3, 4b, and 4c). Lignin was also in yellow color in the bulliform cell walls of *Ph. australis* grown in terrestrial soil (Figures 4d and 4e). The intensity profile of the image along the auremine-*O*-lignin complex in the outer walls of bulliform cells was almost double more than in outer walls of bulliform cells of water *P. australis* plants. Inner walls of bulliform cells of terrestrial plants showed a weak number of lignin fluorescence intensity (Figures 3, 4e, and 4f).

It was shown that syringyl and guajacyl monolignols in the presence of the specific fluorescent indicator diphenyl boric acid-2-aminoethyl ester (DPBA) were in a bright blue color (for syringyl) and green color (for guajacyl) in the bulliform cells of *P. australis* grew in water (Figures 5a–5c). The intensity profile of syringyl was less in the outer walls, whereas the intensity profile of guajacyl was more in the outer walls in comparison to the inner wall of bulliform cells (Figures 5c and 5d). In the presence of the specific fluorescent DPBA, syringyl and guajacyl monolignols have analogical fluorescence in walls of the bulliform cells of *P. australis* grown in terrestrial soil (Figures 5e–5g). The intensity profile of syringyl in the outer walls of motor cells was almost twice lower than that in the inner walls, whereas the intensity profile of guajacyl in the outer walls of the motor cells was seven times higher than the intensity profile of this monolignol in the inner walls (Figures 5g, 5h, and 6).

3.3. Biochemical analyses of polysaccharide in *Phragmites australis* leaves

The present of crystalline and amorphous cellulose, lignin, hemicellulose and pectin was detected in *P. australis* leaves independent of plant location. The determination of the cellulose content of the leaves of the reed growing in water revealed that the total cellulose content was 506 ± 17.13 mg/g FW, of which the amorphous cellulose content was 235 ± 18.16 mg/g and content of crystalline cellulose was 271 ± 16.12 mg/g. The ratio of crystalline cellulose content to amorphous cellulose content was 1.17. The content of other polysaccharides in the leaves of aquatic *P. australis* was the next: lignin – 139 ± 10.1 mg/g DW, hemicelluloses –127 ± 9.7 and water-soluble pectin –87 ± 7.4 mg/g, accordingly.

The next content of polysaccharide was founded in leaves of *P. australis* terrestrial plants: the total cellulose content was 476 ± 31.21 mg/g, amorphous cellulose –181 ± 17.19 mg/g FW, crystalline cellulose –295 ± 31.0, lignin

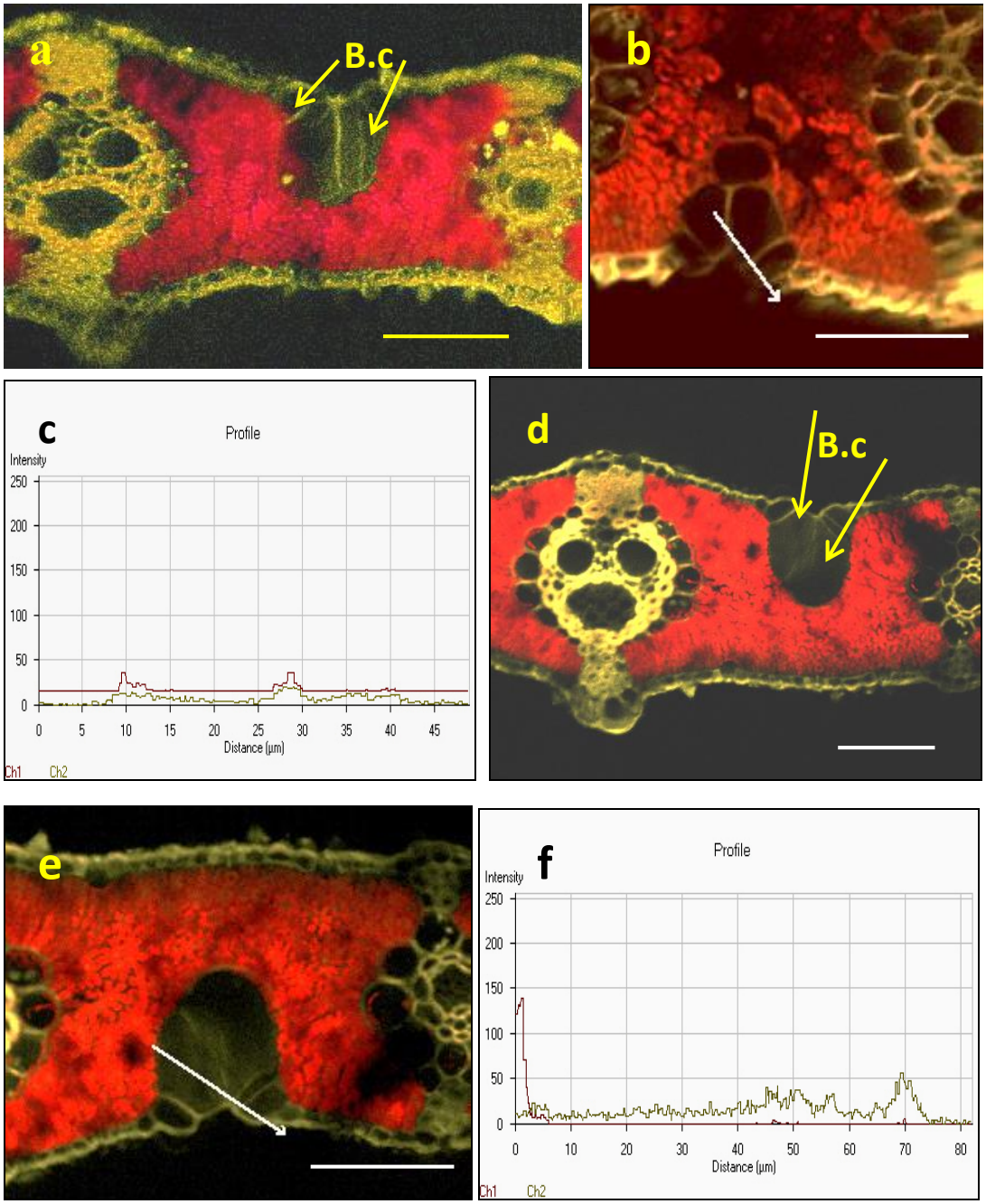

Figure 4. Confocal laser scanning microscopic images of fluorescence lignin in walls of the bulliform cells in leaves of *Phragmites australis*, grown in water (**a, b**) and on terrestrial soil (**d, e**). The leaf sections treated with 0.001% auramine-*O* (**a, b, d, e**) (marker of lignin). Lignin has a yellow fluorescence. In the figures **c** and **f** histograms of intensity profile of lignin (yellow line). Ordinate — intensity of profile, relative units; abscissa — distance was scanned on the **b** and **e**; this distance is indicated as white arrow on the **b** and **e.** Abbreviations: **B.c**: bulliform cells. Scale bars: 100 µm.

–157 ± 9.31; hemicelluloses –108 ± 8.3 and water-soluble pectin 75 ± 5.9 mg/g FW, accordingly. The ratio of crystalline cellulose content to amorphous cellulose content in leaves of terrestrial reed plants was more than that in plants, grown in water (Table 2).

4. Discussion

4.1. Moderate drought determines the morphological response of bulliform cells

The study of the effect of drought on plant ecology is one of the most urgent tasks of modern biology, as this will

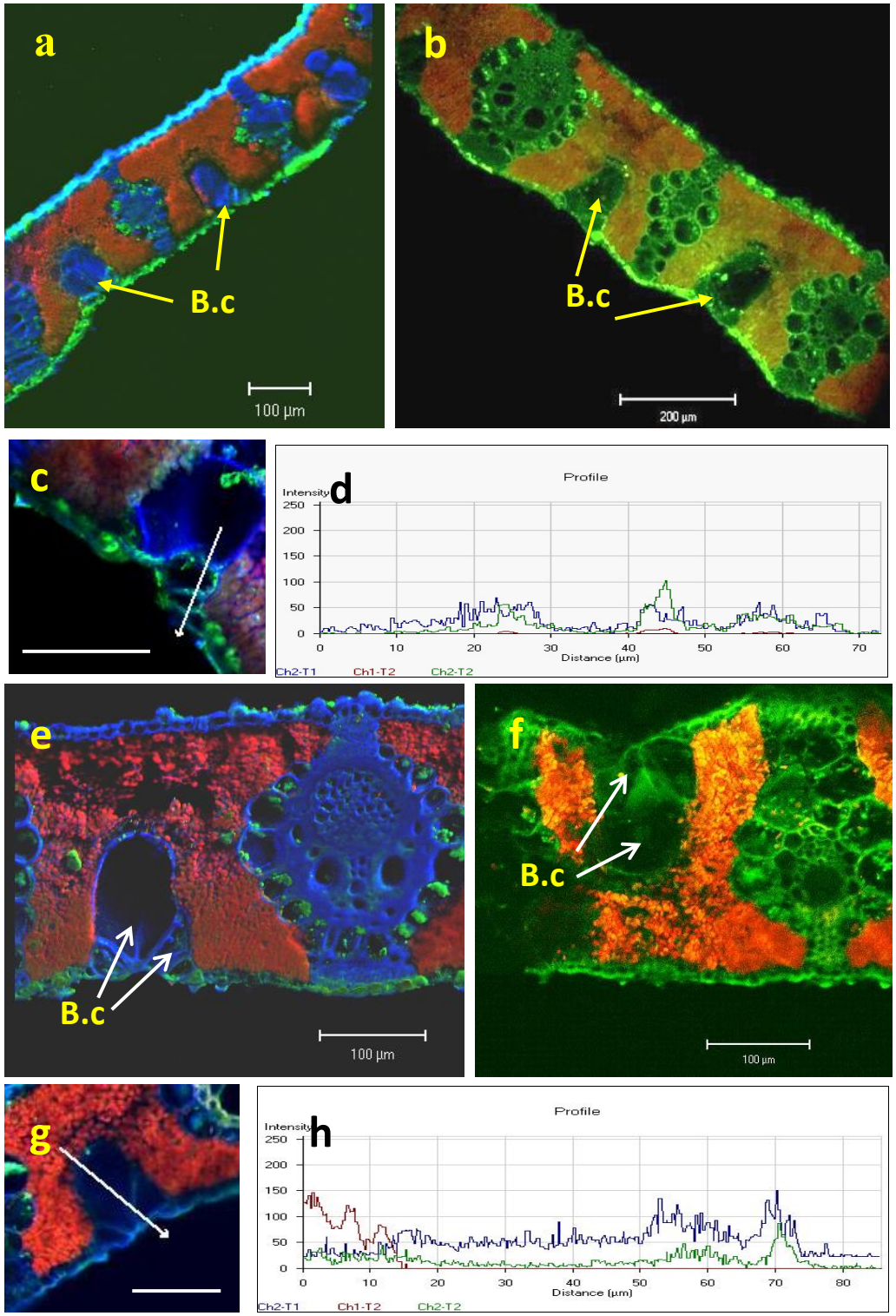

Figure 5. Confocal laser scanning microscopic images of fluorescence monolignols (syringyl and guajacyl) in walls of the bulliform cells in leaves of *Phragmites australis*, grown in water (**a, b, c**) and on terrestrial soil (**e, f, g**). The leaf sections treated with 0.25% solution of diphenyl boric acid-2-aminoethyl ester (marker monolignols). Syringyl has a blue fluorescence, quajacyl — green. In the figures **d** and **h** — histogram of intensity profile of monolignols (blue and green lines, syringyl — blue line and quajacyl — green line). Ordinate — intensity of profile, relative units; abscissa — distance was scanned on the **c**; this distance is indicated as white arrow on the **c** and **g.** Abbreviations: **B.c**: bulliform cells. Scale bars: **a, c, f, e, g** = 100 µm and 200 µm (**b**).

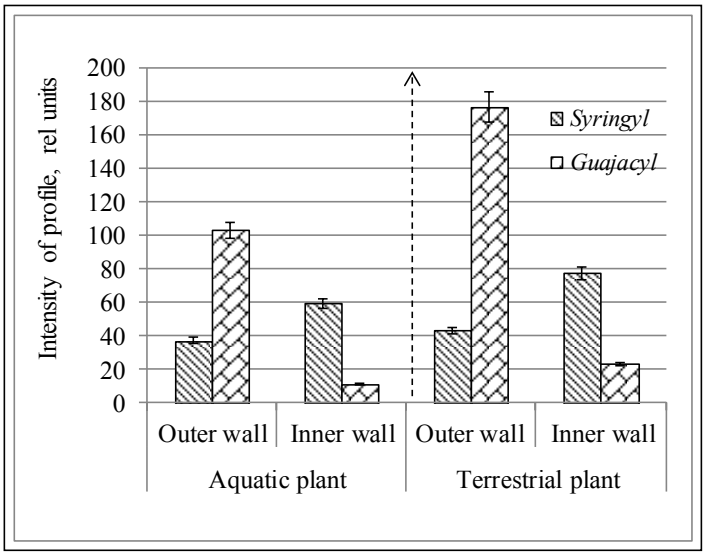

Figure 6. The intensity profile of monolignols in the walls of leaf bulliform cells of *Phragmites australis* grown in water and on terrestrial soil. Data was obtained using the Pascal program and laser confocal microscopy. Statistical significance of relative content (or intensity of profile) of syringyl and quajacyl monolignol in the cell walls were determined using Origin 6.1, including the standard error (means ± SE).

Table 2. Polysaccharides in *Phragmites ausralis* leaves in the vegetative growth stage. Asterisk denotes significant differences in the parameters of aquatic plants from terrestrial plants (* $p \leq 0.05$) (means ± SE).

Polysaccharides	Polysaccharide content (mg/g DW) in leaves of *P. ausralis*	
	Aquatic plant	Terrestrial plant
Total content of cellulose	506 ± 17.13	476 ± 31. 21*
Content of amorphous cellulose	235 ± 18.16	181 ± 17.19*
Content of crystalline cellulose	271 ± 16.1.2	295 ± 31.0*
Content of lignin	139 ± 10.10	157 ± 9.31*
Content of hemicelluloses	127 ± 9.70	108 ± 8.30*
Content of water-soluble pectin	87 ± 7.40	75 ± 5.90*
Content of protopectin	71 ± 5.30	93 ± 8.10*

affect agricultural productivity as well as natural plant populations. One invaluable and long-used method for examining plant drought responses is a comparative study of the morphological, structural, and functional characteristics of plants grown in different water supply conditions (Marchin et al., 2020). During soil drought or extreme temperature rise, the plant activates all possible structural and functional systems of metabolism for survival in unfavorable conditions. A decrease in stomata transpiration, thickening of cell walls, and an increase in deposits of lignin and wax in the cell walls of leaves occurs during drought (Kerstein, 1996). Grigore et al. (2010) reported that the decrease in cuticle and stomata transpiration can also occur by rolling or folding leaves, which leads to a reduction in the area of water evaporation and the area of absorption of light by leaves during drought (Grigore et al., 2010). The bulliform cells of the leaf epidermis take part in rolling the leaf. But investigation of structural-functional signs of these cells under study was poor.

In our comparative study of the leaves of aquatic and terrestrial *Phragmites* australis we found the presence of groups of fan-shaped motor (bulliform) cells in the epidermis placed between the vascular bundles in contact

with the mesophyll, epidermis, and stomata. A similar arrangement of bulliform in the grooves of the epidermis near mesophyll has been described by other researchers in *Bolboschoenus maritimus* (L.) Palla ssp. compactus (Hoffm.) Dobrow, *Carex distans* L., *Carex vulpina* L. (Cyperaceae), *Juncus gerardi* Loisel. (Juncaceae), *Agrostis stolonifera* L., *Alopecurus arundinaceus* Poir. and in *Puccinellia distans* (L.) Parl. ssp. limos (Schur) Jáv (Poaceae) (Grigore et al., 2010; Grigore and Tome, 2014).

The differences in the number of bulliform cells composing one group, namely, more cells (up to 6–7) in leaves of aquatic plants, and fewer cells (from four to five) in the leaves of terrestrial plants of reed were established in our research. Obviously, this phenomenon is a plastic feature of the leaf blade of the plant in response to the change of soil moisture. This is also consistent with the data obtained by us about the decrease in the ratio of the area of motor cells to the area of surrounding mesophyll cells in the leaves of aquatic reed plants compared with those of terrestrial plants (Table 1). The differences were also found in the investigation into the reduction of blade thickness and cell size. Similar differences in the anatomic structure of motor cells and water content in rice leaves are described by other researchers (Liang-Ping Zou et al., 2011). These researchers, by studying the curl genes of rice leaves, found a correlation between leaf humidity, transpiration coefficient, and linear motor cell sizes when comparing these indicators with similar wild-type rice leaf indices. They found clear differences in the structural and functional characteristics of wild-type rice leaves, which had smaller bulliform cells in the group (3–5 cells). Wild-type rice leaves were characterized by lower leaf moisture compared to similar leaf characteristics in rice mutants (oul1), in which the bulliform cells were larger in size and number in one group (6 to 7), and the leaves had higher water content.

It is known that for optimal transpiration and/or reduced visible light surface or absorption of harmful UV radiation, water from motor cells must leave during the twist of leaves (Xiang et al., 2012). It is established that superhydrophilic leaf surfaces cause water leakage on the convex surface of the epidermis. This also occurs with the participation of both water-porous structures and under the conditions of specific structuring of hydrophilic epidermal surfaces of the walls, due to which the hydrophilicity of the leaves increases (Koch and Barthlott, 2009). It is revealed that the coming out of water from a cell over short distances occurs in two ways: diffusion through the plasma membrane of the plasma membrane and with the participation of aquaporins, water channels (Verkman and Mitra, 2000). The rate of transmembrane water flow can be controlled by varying the amount or activity of aquaporins. It can be assumed that some of the water enters across the water channels of the plasma membrane of the bulliform cells into the symplast of surrounding mesophyll cells and stomata adjacent to the motor cells. We also do not exclude that the surface of reed motor cells is characterized by the properties of superhydrophilicity, which is based on the presence of the convex surface of periclinal walls and the presence of microchannels (Koch and Barthlott, 2009). We found no microchannels in the cell walls, but it remains unclear if the cell walls of motor cells respond to external influences by polysaccharide content.

In our research, the differences in content of cellulose, callose, lignin, and monolignols in the bulliform cell walls in the leaves of *P. australis* aquatic and terrestrial plants were revealed. In addition, polysaccharides such as callose and amorphous cellulose are known to be characterized by considerable water permeability (Aidemark et al., 2009).

Callose is known to be a polysaccharide consisting of chains of 1,3- and 1,6-β-glycosidic residues, whose specific role is not only in maintaining the mechanical stability of the walls but also in regulating the transport of water and aqueous solutions between cells (Aidemark et al., 2009). Callose can also accumulate water, swelling several times (Clarke and Stone, 1963). Our results have shown that the content of callose in the cell walls of motor cells in aquatic plants is twice as high as in the leaves of terrestrial plants, and the content of this polysaccharide in the outer walls is also significantly higher than in the inner walls of the bulliform cells. From the known properties of callose in water apoplastic and symplastic transport, it can block this transport only when the content of ionized calcium is increased (Nedukha, 2015), as well as the data obtained on the effect of soil moisture on the content of callose in the bulliform cells, we can make the following assumption:

—With increasing soil moisture, the formation of callose in the cell walls of bulliform cells of *Phragmites australis*, which grows in water, increases;

—The callose distribution in the periclinal and anticlinal (internal) walls changes; the majority of the callose is deposited in the periclinal walls in contact with the environment;

—Callose participates in the water transport of water through the periclinal walls to the outside, as well as through the inner walls to the mesophyll and stomata cells contacting the bulliform cells.

A study of the localization and relative content of monolignols in motor cells revealed the presence of syringyl (S) and guajacyl (G) in both the outer and inner cell walls of the reed leaves, regardless of the soil moisture on which *P. australis* was grown. In the outer walls of the bulliform cells, guajacyl prevailed, whereas in the inner walls, syringyl, respectively. Earlier research into the leaf surface and also the presence of monolignols in different types of reed leaf epidermal cells revealed that the periclinal

walls of ordinary cells (neither trichomes nor stomata) in the leaf surface valley contained three times more guajacyl than identical cells of the reed terrestrial ecotype (Nedukha, 2021).The intensity profile of monolignols syringyl and guajacyl and the ratio of these monolignols are known to influence the mechanical ability of the wall: a decrease in the S/G ratio weakens the mechanical stability of the cell walls and lowers the water penetration barrier (Leisola et al., 2012). In addition, it has been established that the main function of guajacyl monolignol is to create flexibility and softness in the walls (Menden et al., 2007). Considering the experimental data obtained regarding the presence of monolignols and their different content in the cell walls of bulliform cells of the studied samples of reed leaves, and the above data regarding the functional value of monolignols, we can assume:

— When folding reed leaves (irrespective of ecotype), the presence of guajacyl is functionally necessary for rapid flattening and reduction of mechanical stability of the outer walls of bulliform cells when water comes out;

— The presence of a high content of syringyl in the inner walls of the bulliform cells in contact with mesophyll cells contributes to the mechanical stability of the inner part of the leaf blade at the border of mesophyll-motor cells;

– An increase in the content of guajacyl monolignol in the outer walls of bulliform cells in reed terrestrial plants' leaves aids in leaf twisting.

In this study, it was shown that the ratio of amorphous to crystalline cellulose in *Phragmites australis* leaves depended on the location of plants. The content of amorphous cellulose in the leaves of aquatic plants was higher than in terrestrial plants. The higher content of crystalline cellulose in the leaves of terrestrial plants and the higher content of amorphous cellulose in the leaves of reed aquatic plants can be explained as follows. Early on, it was established that changes in the content of amorphous and crystalline cellulose in a plant depend on the conditions of its growth (Heinze, 2016). It is known that amorphous cellulose molecules respond to changes in osmotic pressure in the cell; this cellulose is capable of adsorbing water and passing it between adjacent molecules, which promotes apoplastic water transport, whereas crystalline cellulose does not have this property (Nilsson, 2006). It is known that water is absorbed by amorphous cellulose, in which hydrogen bonds are dominated, and a crystalline form of cellulose is not involved in the transport or absorption of water molecules. The first stage is the adsorption of water molecules with one molecule chain of amorphous cellulose; the second stage is the binding of water molecules between chains of cellulose molecules in one microfibril; noticeably, water is absorbed between the two chains of amorphous cellulose; and the third stage is when the water molecules join to other water molecules that are linked to amorphous cellulose microfibrils (Nilsson, 2006). This phenomenon suggests that amorphous cellulose is a sensitive polysaccharide of the cell wall, which reacts to the water environment by activation of its synthesis and accumulation that probably promotes the storage of water as it passes through the apoplast. The increase of amorphous cellulose in the leaf cell walls of *P. australis* may be an evidence for polysaccharide adaptation in induction of amorphous cellulose synthesis and/or change of activity of a complex of cellulose synthase enzymes under the influence of an unknown inducer. Allele RSW1 responsible for the synthesis of amorphous cellulose was determined by genetic studies of Petunia hybrida wild plants and mutant plants, and Petunia hybrida is characterized by a mutant gene for PhEXP1 (Zenoni et al., 2004).

In addition, the periclinical walls of leaf bulliform cells in the species of *Loudetiopsis chrysothrix* (Nees) Conert and *Tristachya leiostachya* Nees (Poaceae) were very thin and contained very few cellulose microfibrils (Alvarez et al., 2008). Given the above literature data and our experimental results regarding the intensity of cellulose fluorescence in periclinal and anticlinal walls of reed leaf motor cells and also biochemical data on the presence of amorphous cellulose in our reed samples, we can assume that its content should be greater in the bulliform cells of aquatic plants than in the leaves of terrestrial plants. This can be explained not only by the different content of amorphous cellulose but also by the higher water content of the leaves of water reeds. Obviously, this is necessary for the absorption of water by the walls of bulliform cells, and the exit of water from these cells onto the outside of the leaf and inside of adjacent mesophyll and stomata cells in contact with motor cells. We believe that the presence of amorphous cellulose in reed bulliform cells is also necessary to maintain optimal water balance in adjacent cells when leaf twisting under adverse conditions, both in aquatic and terrestrial reed plants.

The presence of more hemicellulose content in the leaf cell walls of aquatic reed in comparison with that in terrestrial plants may indicate a change in the water status of cells. Some properties of hemicelluloses are similar to those of amorphous cellulose, because both hemicelluloses and amorphous types of cellulose have a high water-retaining ability. This property of hemicelluloses is based on the fact that the area of their hygrophilous surface, in particular xylan, is considerably larger than that of the other polysaccharides (Ress and Sternberg, 1998). The question of the optimal ratio of the content of hemicelluloses, pectins, amorphous and crystalline cellulose in the bulliform cell walls, which optimizes their function when twisting or rolling leaves under adverse conditions, is open and requires further study.

5. Conclusion

1. The cytological and cytochemical studies of bulliform cells in leaves of *Phragmites australis* grown in water and in terrestrial soil have shown significant differences in the structural-functional characteristics of epidermis bulliform cells. The differences in the number, size, and area of bulliform cells and also changes in wall polysaccharide content show clear phenotypical plasticity and may reflect modification of bulliform cells that occurred in response to specific environmental challenges.

2. Cytochemical and laser confocal microscopic studies of cellulose, callose, lignin, and monolignols in the wall bulliform cells of water and terrestrial reed plants revealed that a decrease in soil moisture leads to an increase in lignin and syringyl monolinol content in the outer walls of bulliform cells. The change in the ratio of monolignols in bulliform cell walls is considered a sign of the plasticity of these cells, which promotes the folding of the leaves under adverse conditions of the water regime. The decrease of amorphous cellulose was revealed in the leaves of terrestrial plants. We consider that the amorphous form of cellulose contributes to the function of bulliform cells when folding leaves, regardless of soil moisture. We assume that the change of the polysaccharides in the walls of bulliform cells leads to a decrease in cuticular transpiration and to optimization of the water balance of plants during a twisting leaf.

3. The obtained data shows that the studied signs of cell walls can be markers of tolerance plant that have the ability to curl leaves in moderate drought with preservation of optimal water statement.

Acknowledgments

This work was financially supported by the National Academy of Science of Ukraine; the studies were performed in the Department of Cell Biology and Anatomy of Institute of the Botany of National Academy of Sciences of Ukraine (No. 0110U000087 – state registration number; theme No. 432 – Resistance to the ontogeny of plants of different ecological natures to water stress: cellular and molecular aspects). The author did not receive any financial support from any organization to assist with the preparation and for the submission of this manuscript.

The author is grateful to Ms. Victoria Ivacha for expert technical help during the study of the plant samples with the laser confocal scanning microscopy for completing theme No. 432.

Conflicts of interests

The author declares no conflict of interest.

References

Aidemark M, Andersson C–J, Rasmusson AG, Widell S (2009). Regulation of callose synthase activity *in situ* in alamethicin-permeabilized *Arabidopsis* and tobacco suspension cells. BMC Plant Biology 9, 27: 1–13.

Alvarez JM, Rocha JF, Machado SR (2008). Bulliform cclls in *Loudetiopsis chrysothrix* (Nees) Conert and *Tristachya leiostachya* Nees (Poaceae): Structure in Relation to Function. Brazilian Archives of Biology and Technology An Internat Journal 51: 113-119.

Arasimovich VV, Ermakov AI (1987). The determination of polysaccharides and lignin. In: Methods of Biochemical Study of Plants. Ed. Ermakov AI. Leningrad, Agropromizdat. Page 143-172. (In Russ.).

Brix H (1999). Genetic diversity, ecophysiology and growth dynamics of reed (*Phragmites australis*). Aquatic Botany 64: 179–184.

Candela H, Johnston R, Gerhold A, Foster T, Hake S (2008). The *milkweed pod 1* gene encode a KANADI protein that is required for abaxial/adaxial patterning in maize leaves. Plant Cell 20: 2073-2087.

Clarke AE, Stone BA (1963). Chemistry and biochemistry of β-1, 3-glucans. Reviews of Pure and Applied Chemistry 13:134-156.

Currier HB, Shin CY (1968). Sieve tuber and callose in *Ellodea* leaves. American Journal of Botany 55: 145-152.

Dickison WC (2000). Integrative Plant Anatomy. Harcount Academic Press. San Dieg, San Francisco, NY, Boston, London, Toronto, Sydney, Tokyo. 1-533 p.

Djikanovic D, Kalauzi A, Radotic K, Lapierre C, Jeremic M (2007). Deconvolution of lignin fluorescence spectra: a contribution to the comparative spectral studies of lignin.(Журнал физической химии (naukapublishers.ru)).Russian journal of Physical Chemistry) 81: 1425-1429.

Eller F, Xiao Guo, Siyuan Ye, Mozdzer TJ, Brix H.1 (2020). Suitability of wild *Phragmites australis* as bio-Resource: tissue quality and morphology of populations from three continents**.** Resources 9: 143.

Ermakov AI (1982). Determination of water content and active acidity plant objects. In: Methods of Biochemical Research of Plants. Ed. A. Ermakov: Agropromizdat: 20-35. Leningrad, VO. (In Russ.).

Esau K (1969). Plant Anatomy. Ed. L. Kudrjashov. Moscow, Publ. Mir, 564 p. (UDK 581.4. In Russ.). Translation from second English edition: John Wley and Sons, Inc., New York – London – SydneY.

Grigore MN, Toma C (2011). Ecological implafications of bulliform cells on halophytes, in water stress natural conditions. Studia Universitatis "Vasile GoldişUniversity", Seria Ştiinţele Vieţii 21: 785-792

Grigore MN, Toma C (2014). Intergrative ecological notes of halophytes from "Valea ilenet" (Lasi) nature reserve. Memoirs of the Sci. Sections of the Romanian Academy 37: 19-37.

Grigore MN, Toma C, Boscaiu M (2010). Ecological implications of bulliform cells of Halophytes, in salt and water stress natural conditios. Analele ştiinţifice ale Universităţii "Al. I. Cuza" Iaşi. II a. Biologie vegetală LVI: 5-15.

Heinze T (2016). Cellulose: structure and properties. Advances in Polymer Science 271: 1-52.

Herth W (1980). Calcofluor white and congo-red inhibit microfibril assembly of *Poteriochro-monas*: evidence for a gap between polymerization and microfibril formation. Journal of Cell Biology 84: 642-658.

Hong MG, Nam BE, Geun Kim JG (2019). Differences in functional traits of leaf blade and culm of common reed in four habitat types. Journal of Ecology and Environment. 43, article N12: 1-6.

Jenson WA (1965). Botanical Histochemistry. Principes and Practice. Moscow, Publ. Mir. UDK.581.1

Kadi SA, Ouendi M, Ouendi C, Berchiche M, Gidenne T (2018). Nutritive value of sun-dried common reed (*Phragmites australis*) leaves and its effect on performance and carcass characteristics of the growing rabbit. World Rabbit Science 26: 113-121.

Kadioglu A, Terzi R (2007). A dehydration avoidance mechanism: leaf rolling. The Botanical Review 73 (4): 290–302.

Köbbing JF, Thevs N, Zerbe S (2013). The utilisation of reed (*Phragmites australis*): a review. Mires Peat 13: 1–14.

Kerstein G (1996). Cutiular water permeability and its physiological significance. Journal of Experimental Botany 47:1813-1832.

Koch K, Barthlott W (2009). Superhydrophobic and superhydrophilic plant surfaces: an inspiration for biomimetic materials. Phylosophical Transactions of Royal Society A. 367: 1487-1509.

Kogan, FN (1997). Global drought watch from space. Bulletin of the American Meteorological Society 78 (4): 621–636.

Leisola M, Pastinen O, Axe DD (2012). Lignin— designed. BIO-Complexity 3: 1-11.

Li L, Shi Z-Y, Li L, Shen Z-Zh, Wang X-Q (2010). Overexpression of ACL1 (abaxial curved leaf 1) increased buliform cells and induced abaxial curling of leaf blades in rice. Molecular Plant 3: 807-817.

Liang-ping Zou, Xue-hui Sun, Zhi-guo Zhang, Peng Liu, Jin-xia Wu et al. (2011). Leaf rolling controlled by the homeodomain leucine zipper class IV gene *Roc5 i*n Rice. Plant Physiology 156: 589–1602.

Mahmoud A Elhaak , Awatef A Mohsen , El-Sayed AM, Hamada Fathy E, El-Gebaly (2015). Biofuel production from *Phragmites australis* (Cav.) and *Typha domingensis* (Pers.) plants of Burullus lake. Egypt. Journal of Experimantal Botany 11: 237-243.

Marchin RM, Ossola A, Leishman MR, Ellsworth DS (2020) A Simple method for simulating drought effects on plants. Frontiers in Plant Science 10: 1-14.

Menden B, Kohiholf M, Moerschbacher BM (2007). Wheat cell accumulation a syringyl-rich lignin the hypersensitive resistance response. Phytochemistry 68: 513-529.

Nedukha OM (2015). Callose: localization, function and synthesis in plant cells. Cytology and Genetics 49: 49-57 (In Ukr.).

Nedukha OM (2021). Micromorphology and monolignols of leaf epidermis in *Phragmites australis* (Poaceaea) of air-aquatic and terrestrial ecotypes. Protoplasma 258: 949-961.

Nilsson M (2006). Water-induced charge transport in microcrystalline cellulose. Acta Universitatis Upsaliensis. Uppsalla 170:1-54.

Park HJ, Nam BE, Hong MG, Kim JG (2018). Slope and soil nutrients can explain the distribution of *Phragmites australis* and *Phragmites japonica* in riparian wetlands. River Research and Application 34:1229–1233.

Pausheva ZP (1988). A workshop on Plant Cytology. Publisher: Moscow, Agropromidat.

Price AH, Young EM, Tomos AD (1997). Quantitative trait loci associated with stomatal conductance, leaf rolling and heading date mapped in upland rice (*Oryza sativa*). New Phytologist 137:3–91.

Ress E, Sternberg E (1998) From Cells to Atoms. Moscow, Publ.: Mir. (In Russ.).

Ricardo MF. da Costa1, Winters A, Hauck B, Martín D et al. (2021). Biorefining potential of wild-grown *Arundo donax*, *Cortaderia selloana* and *Phragmites australis* and the feasibility of white-rot fungi-mediated pretreatments. Fronties in Plant Science 12: Article 579966.

Smith CA, Skvirsky RC, Hirsch A (1986). Histochemical evidence for the presence of a suberinlike compound in Rhizobium-induced nodules of the nonlegume *Parasponia rigida*. Canadian Journal of Botany 64: 474-1483.

Verkman AS, Mitra AK (2000). Structure and function of aquaporin water channels. American Journal of Physiology - Renal Physiology 278: 13-28.

Weakley BS (1975). A Beginners Hand book in Biological Electron Microscopy. Moscow, Publ Mir, 228 p. (УДК 591.8.611. In Russ.).

Weber DJ, Rasmussen HP, Hess WM (1977). Electron microprobe analyses of salt distribution in the halophyte *Salicornia pacifica* var. Utahensis. Canadian Journal of Botany 55: 1516–1523.

Wuyts N, Lognay G, Swennen R, De Waele D (2005). Secondary metabolites in roots and implications for nematode résistance in banana (*Musca s*p.). In: Banana Root System: towards a better understanding for its productive management. Eds. Turner D and Rosales F. page 238-246.

Zenoni S, Reale L, Tornielli GB, Lanfaloni L, Porceddu A et al. (2004). Downregulation of the *Petunia hybrida* α-expansin gene *PhEXP1* reduces the amount of crystalline cellulose in cell walls and leads to phenotypic changes in petal limbs. The Plant Cell 16: 295–308.