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CHARACTERISTICS OF PHOTOSYNTHETIC APPARATUS OF AQUATIC FERN *SALVINIA NATANS* FLOATING AND SUBMERGED FRONDS

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Abstract. We analyzed chloroplast ultrastructure, microstructure of fronds surfaces, pigment`s content and efficient use of light by photosynthetic apparatus in floating and submerged fronds of water fern *Salvinia natans* at different stages of development. In parenchymal cells of floating fronds average chloroplast cross-section area at intensive growth stage (June) was 5.56 μm^2. Well-developed system of thylakoid membranes formed numerous grana. Up to four starch grains occurred. In submerged fronds chloroplasts cross-section area was 2.16 μm^2. Degradation of grana thylakoid membrane and disruption of intergranal connections were revealed in fronds in stage of sporocarp formation (September). Rows of plastoglobules were found in chloroplasts of floating frond. Starch grains were almost entirely absent in stroma. Adaxial surface of floating fronds had simple structure stomata. The abaxial surface had no stomata and the cuticle was not developed. Pigment`s content in floating fronds was twice higher than that of submerged ones. During development of fern a content of pigments raised up in the floating leaves. At stage of sporocarps formation the chlorophyll and carotenoid content reduced. Floating fronds showed an effective operation of chloroplast photosynthetic electron-transport chain. Submerged fronds exposed to intensive light were characterized by decrease of efficiency of PSII and increase of non-photochemical quenching of chlorophyll fluorescence.

Keywords: *cell ultrastructure, chlorophyll fluorescence, ontogenesis, Salvinia natans (L.) All.*

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Abbreviations

F_v/F_{max} – maximum quantum efficiency (yield) of PSII photochemistry
NPQ – non-photochemical quenching
qP – coefficient of photochemical quenching
r. u. – relative units
RuBisCO – D-Ribulose-1,5-bisphosphate carboxylase oxygenase
SEM – scanning electron microscopy
$\Phi_{(PSII)}$ – effective quantum yield of PSII

1. Introduction

Salvinia natans (L.) All. is an aquatic floating heterosporous fern of the family *Salviniaceae*. It is widespread in tropical, subtropical, and temperate climate regions of the world. Morphological characteristics of the plant make it intriguing and unique among ferns. The plant has a summer-green phenotype and occurs at the air-water boundary forming floating and submerged fronds (Smith *et al.*, 2006). Close to the base of submerged fronds, bullet-shaped sporocarps are formed in clusters of 4-5. Submerged

leaves are distinct in anatomic structure and morphologically are similar to roots (Bercu, 2006). However, a number of studies have indicated that submerged fronds are initiated in the stem apical meristem as one of the three leaf primordia per each node. Two of the leaf primordia develop in floating fronds while the third one forms a multi-segment, root-like structure (Croxdale, 1978, 1981; Lemon *et al.*, 1997; Preston and Ackerly 2004; De Kroon *et al.*, 2005; Barthlott *et al*., 2009). Submerged fronds of floral hydrophytes are known to have a lower content of photosynthetic pigments compared to above-water and floating leaves (Ryen, 1985; Yang *et al*., 1987) and, accordingly, lower productivity (Sand-Jensen *et al.*, 1999). To date, most of chloroplast ultrastructure studies were carried out on flowering hydrophytes (Kordyum and Klimenko, 2013; Nedukha, 2013). But a detailed electron-microscopic analysis of organelles and photosynthetic pigments content in floating and submerged fronds of fern-hydrophytes in ontogenesis is yet to be done. Still to be studied are such characteristics of photosynthetic apparatus as chlorophyll fluorescence which enables to estimate the functional condition of chloroplast pigment-protein complexes and the effectiveness of utilization of light energy of various intensities (Maxwell and Johnson, 2000; Rohacek et al., 2008; Brestic and Zivcak, 2013). In our studies we assumed that the adaptation of *S. natans* to the existence at the air-water boundary depends among other factors on features of the structure and functions of its photosynthetic apparatus. Therefore, the aim of our work was to conduct a comparative study of structural-functional peculiarities of the photosynthetic apparatus in floating and submerged fronds at various stages of ontogenesis of the water fern *S. natans*, and to find functional adaptations that enable ferns to successfully exist at the water-air interface.

2. Materials and methods

The study was conducted with *S. natans* sampled in ponds of Kyiv city in 2014-2015. The surface of the pond was not shaded. Sun light intensity on the surface of the pond reached the level of 1235 $\mu M \times m^{-2} \times s^{-1}$ (65 kLux) at noon. The plant material for the determination of pigments and chlorophyll fluorescence measurement was collected in mid-June (the first investigated stage of ontogenesis), in the second decade of July (the second investigated stage) and in mid-September (the third investigated stage). Chloroplast ultrastructure was studied from the material collected at the first and third stages. The first two stages coincide with active vegetative growth of stem and fronds of *S. natans*. During the third stage, active sporocarp formation occurred at the base of submerged fronds, along with early dissolution of vegetative organs (fronds turned yellow).

Tissue sections measuring 3 mm×1 mm that were cut between the edge and the central rib of floating fronds and 5 mm long sections of cut segments of submerged fronds were fixed with pH 7.2 buffered solutions of 3% glutaraldehyde and postfixed with 1% osmium tetroxide, dehydrated in a series of ethyl alcohol solutions at increasing concentrations, and after acetone treatment placed in a mixture of epoxy resins Epon and Araldit according to the method (Carde, 1987; Shcherbatiuk *et al*., 2015). Ultrathin sections were analyzed using the transmission electron microscope JEOL JEM-1230. For the SEM, fresh fronds were frozen in liquid nitrogen then lyophilized and studied after gold coating. The scanning electron microscope JEOL JSM-6060LA was used to investigate microstructure of *S. natans* organ surface. Structure sizes were measured with the ImageJ 1.49v.

Photosynthetic pigments were extracted with 80% acetone and were determined according to the method (Wellburn, 1994). Chlorophyll's functional condition was identified using fluorimeter XE-PAM, with real time data recorded using a computer equipped with a special analog-digital converter. Obtained data were used to calculate the maximum (F_v/F_{max}) and effective (Φ_{PSII}) quantum yield (efficiency), photochemical (qP) and non-photochemical quenching (NPQ) according the formulas (Brestic and Zivcak, 2013; Maxwell and Johnson, 2000). Biological analyses were repeated twice and analytical examinations thrice. Obtained data were statistically processed using the Excel program of the standard Microsoft Office 2013 package. Data adequacy was checked according to the t-test applying 5% significance level ($P \leq 0.05$).

3. Results and discussion

Our studies showed that there are distinct differences in the chloroplast ultrastructure of floating and submerged fronds. In July, chloroplasts in parenchyma cells of floating frond had elongated ellipsoid shape and well-developed system of thylakoid membranes that formed numerous grana. The latter were uniformly distributed in the stroma, thylakoids of grana were well-developed and fit tightly to each other, and their boundaries were quite clear and electron-dense (Fig. 1a). In the stroma a significant number of extended starch grains and insignificant number of osmiophilic structures (plastoglobules) were observed (Fig. 1a).

The size of parenchyma cell chloroplasts in submerged fronds was significantly smaller as compared to that of floating fronds (Fig. 1b). Thylakoid membranes were characterized by the highest electron density and fuzzy boundaries. Formation of small starch grains and individual plastoglobules sometimes occurred.

The main ultrastructure morphometric features of mesophyll cell chloroplasts are given in the Table 1. The photosynthetic organelles of floating and submerged fronds clearly differ not only in their size but also in their grana area and number of thylakoids per grana. Submerged frond chloroplasts were shown to have a considerably reduced number of starch grains in the stroma, and their area decreased.

Table 1. The morphometrical characteristics of *Salvinia natans* chloroplasts at the first investigated stage

Parameter	Variant	
	Floating fronds	Submerged fronds
The average length of chloroplasts (long axis), µm	6.59±0.27	3.06±0.33
The average width of chloroplasts (short axis), µm	1.41±0.14	0.92±0.16
Area of one chloroplast section, μm^2	5.56±0.51	2.16±0.28
The average amount of grana on the section of one chloroplast	24.50±1.17	18.83±1.78
The average amount of thylakoids per granum	6.54±1.52	4.30±1.04
The average area of one granum, μm^2	0.038±0.011	0.026±0.009
The average amount of starch grains per chloroplast section	4.11±0.48	0.81±0.48
Area of starch grains per chloroplast section, μm^2	1.63±0.23	0.14±0.10

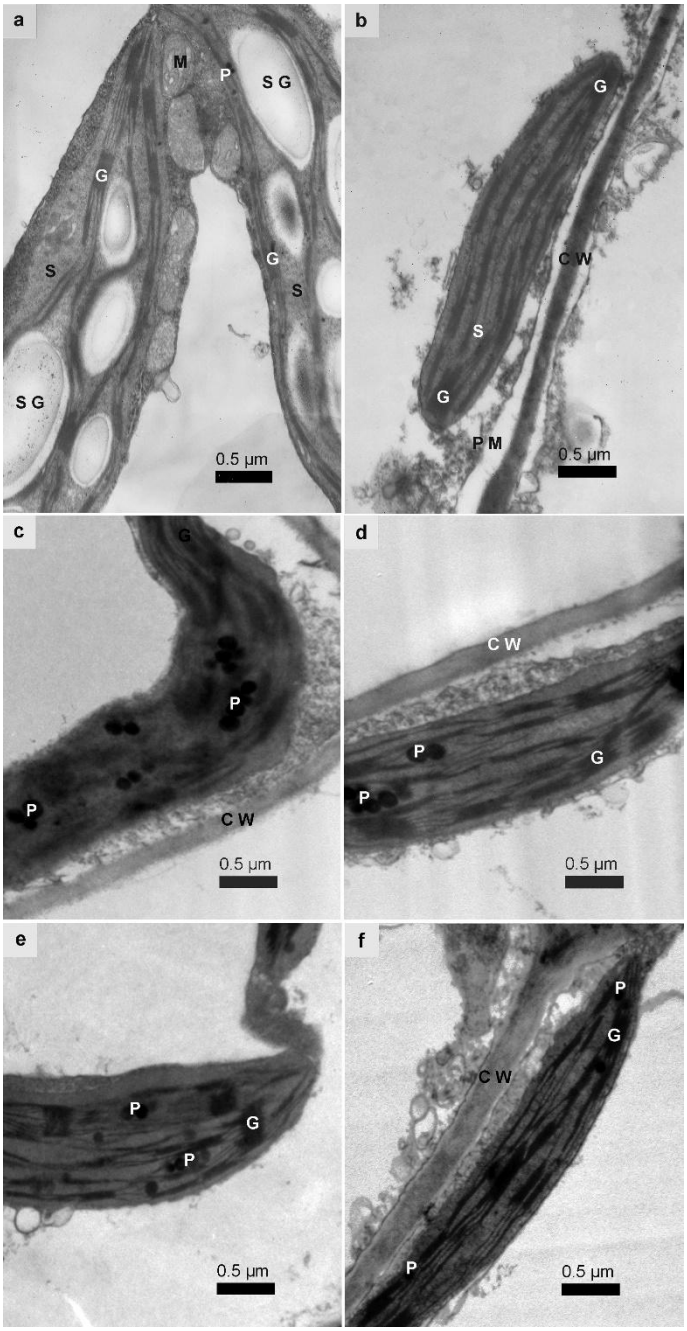

Fig. 1. Transmission electron microscopy microphotographs of cell organelles of *S. natans*: a – the chloroplasts in parenchyma cells of floating fronds at the first investigated stage; b – chloroplast in parenchyma cells of submerged fronds at the first investigated stage; c – chloroplast with partly destroyed membranes in parenchyma cells of floating fronds at the third investigated stage; d – chloroplast with intact membranes in parenchyma cells of floating fronds at the stage of sporocarps at the third investigated stage; e – chloroplasts with partly destroyed membranes in parenchyma cells of submerged fronds at the third investigated stage; f – chloroplast with intact membranes in parenchyma cells of submerged fronds at the third investigated stage. ×15000. Abbreviations: G – grana; SG – starch grain; CW – cell wall; P – plastoglobule; PM – plasma membrane; S – stroma.

Some differences in the submerged frond chloroplast structure most probably associated with conditions of continuous shading caused by floating fronds were revealed. Other studies have indicated that such differences are typical of under-water

leaves in flowering hydrophytes (Kordyum and Klimenko, 2013; Nedukha, 2013; Nielsen, 1993). Our data indirectly confirm that the main producer of assimilates during an intensive sporophyte growth is the floating frond while photosynthetic organelles of the submerged frond provide only an insignificant part of the primary synthesis products.

In September, destructive changes were revealed in the structure of both frond types. Degradation of grana thylakoid membranes and disturbances in intergranal connections were found (Fig. 1c, 1e). The stroma had practically no starch grains. Some swelling of external membranes, changes in stroma density and structure of the intergranal lamellar membrane system were observed. In chloroplasts of floating frond plastoglobules were arranged in rows that preserved contacts with stroma lamellas (Fig. 1c). The emergence of a great number of plastoglobules attests to a high oxidation level induced by highly intensive light effects and higher temperature (Austin *et al.*, 2006; Bréhélin *et al*., 2007; Spicher and Kessler, 2015). We think that this overabundance of a great number of plastoglobules at that final stage of sporophyte ontogenesis corresponds to the active fern aging and coincides with the time of chloroplast reutilization that occurs during the formation of sporocarps with micro- and macrospores.

Thus, the sporocarp formation stage depends on the transformation of 70% of chloroplast population into structures that both partially or completely lose their photosynthesis ability and structurally are similar to chromoplasts. However, the occurrence of about 30% of intact chloroplasts in parenchymal cells of floating and submerged fronds is evidence of retained photosynthetic activity required for maturation of sporocarps with spores (Fig. 1d, 1f).

The surface of floating and submerged fronds of *S. natans* at the first ontogenetic stage was studied using the scanning electron microscope. Adaxial surface of floating fronds contains stomata of simple structure. Stoma openings are located below the epidermis. The number of stomata per 1 mm^2 amounts to 142. Guard cells are sunk in the epidermis, they are partially seen. An average length of stoma opening is 8.1 μm (Fig. 2a). Surface of epidermis cells are honeycomb-like, covered with wax layer and containing rows of trichomes, which have hydrophobic properties except apical cells (Barthlott *et al*., 2009, 2010). The epidermis of frond abaxial surface is covered with hydrophilic trichomes more densely than adaxial one (Fig. 2b). No stomata occur here and the cuticle is practically undeveloped. Due to trichomes the abaxial surface contact area with water increases considerably, stabilizing plant flotation (Fig. 2c).

The epidermis of submerged fronds is formed of thin-walled cells without depositions, ensuring their absorptive function (Fig. 2d). Submerged fronds have no cuticle and are branchy and as a result, the area of their contact with water increases.

The success of adaptation to the environment to a great extent depends on an optimal functioning of the assimilation apparatus, whose state is indicated by the content and ratio of photosynthetic pigments. Biochemical analysis of floating and submerged fronds at different stages of ontogenesis showed the presence of chlorophylls *a* and *b*, and carotenoids.

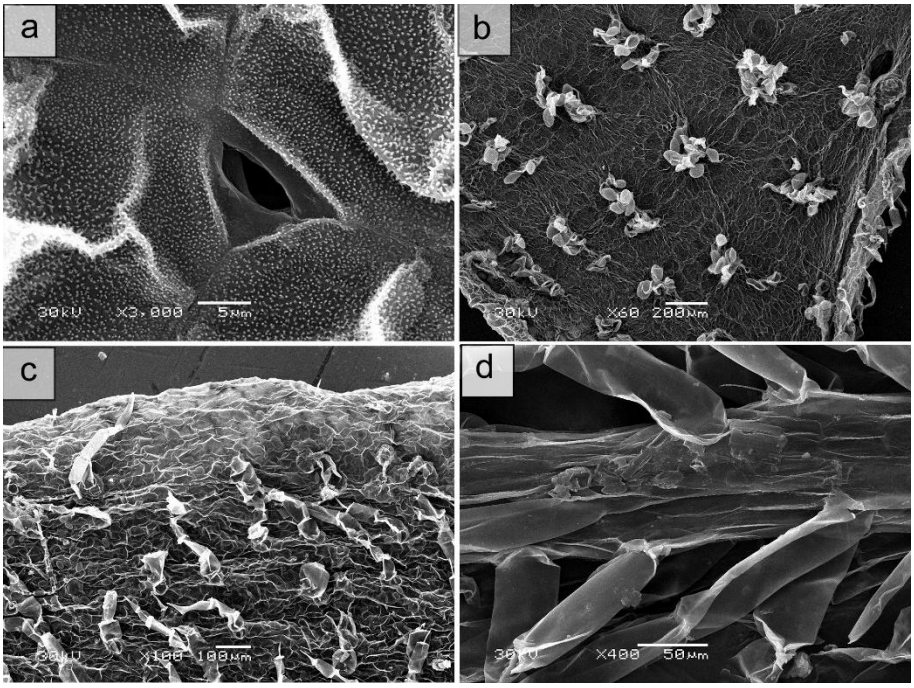

Fig. 2. Scanning electron microscopy microphotographs of *S. natans*: a - stomata on the adaxial surface of floating fronds; b – the adaxial surface of floating fronds; c – the abaxial surface of floating fronds with numerous trichomes; d – surface of submerged fronds.

The content of photosynthetic pigments in floating fronds was two times and at some stages three times higher than that of submerged ones (Fig. 3a, 3b). The ratio of chlorophyll *a* and chlorophyll *b* as well as total amount of chlorophylls were found to be definitely lower in submerged fronds (Table 2). It should be noted that floating fronds actively photosynthesize throughout the whole vegetation period, and are brightly green in color due to high content of chlorophyll *a*. During the first two studied stages the color of submerged fronds as a result of a low chlorophyll content and relatively high concentration of carotenoids was light brown-green (Fig. 3b). At the stage of sporocarp formation the amount of chlorophyll and carotenoids in floating fronds increased while in submerged ones it decreased (Fig. 3a, 3b). During the second stage of sporophyte growth the photosynthetic pigments content in submerged fronds increased. It is significant that as compared to the tissue of leaves of ground flowering plants (Babenko *et al.,* 2014), floating and submerged fronds of *S. natans* contain considerably less chlorophylls *a* and *b*. A lot of hydrophytes were found to have small amounts of photosynthetic pigments and it is regarded as an adaptation to prevent cell degradation due to photo-oxidation (Nedukha, 2013).

As known the main photosynthetic pigment pool is localized in pigment-protein complexes of grana thylakoids (Evert, 2007; Croce and Van Amerongen, 2014). In this connection, the dynamics of pigment content are completely correspondent to the results of ultra-structural studies. Thus, chloroplasts of floating fronds have a many grana that are composed of 4-7 thylakoids and the size of formed starch grains is rather large. The amount of chloroplasts in mesophyllous cells of submerged fronds was low and their grana had a smaller area that coincided with low content of photosynthetic pigments. Like underwater leaves of flower water plants, submerged fronds exhibit modification in the light-harvesting complex on the account of reduced chlorophyll *b*

and carotenoids as adaptation to continuous shading. Submerged fronds have significantly decreased number of chloroplasts that contain starch grains. It was reported about a reliable decrease of carbon isotope inclusion in starch of chloroplasts of hydrophytes submerged leaves (Nekrasova *et al*., 2003). Specific features of *S. natans* submerged fronds turned out to be the more than twice reduced total chlorophylls content (*a*+*b*) and the low *a*/*b* ratio. Such features are typical of plants that grow in shade caused by poor lighting conditions (Nekrasova *et al.*, 1998; Evert, 2007).

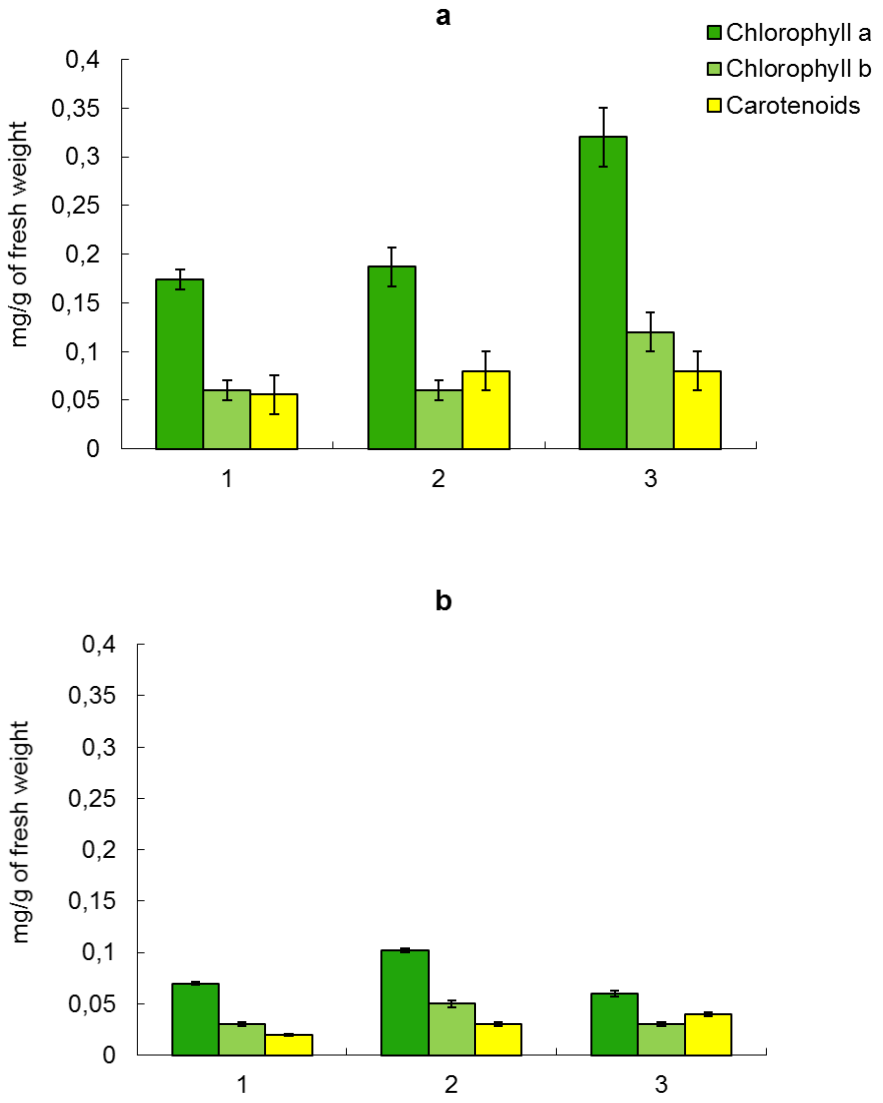

Fig. 3. The content of pigments in floating (a) and submerged (b) fronds of *S. natans* at the different stages of ontogenesis: 1 – the first investigated stage; 2 – the second investigated stage; 3 – the third investigated stage.

Table 2. The ratio of pigments classes at the different stages of *S. natans* ontogenesis

Fronds	*a+b*	*a/b*	*a+b*/car.
first investigated stage			
Floating	0,23	2,90	4,18
Submerged	0,10	2,30	5,00
second investigated stage			
Floating	0,25	3,10	3,09
Submerged	0,15	2,04	5,00
third investigated stage			
Floating	0,43	2,60	5,40
Submerged	0,09	2,00	2,25

Studies on effectiveness of light energy utilization revealed differences in the photosynthetic apparatus functioning both at the level of fern organs and depending on ontogenesis stages. The maximum quantum efficiency (yield) of photochemical reactions PSII (F_v/F_{max}) characterizes the degree of its being intact of photosynthetic membranes. Changes in PSII activity occur during photoinhibition caused by photosynthetic apparatus (PSA) failing to adapt to light effects of certain intensity and sometimes also due to a pathology, drought etc. (Bertic and Zivsak 2013; Maxwell and Johnson 2000). The maximum possible level of the quantum efficiency – 0.823 characterizes an optimal state of PSA, while its values lower than 0.5 testify of deep damages (Johnson *et al.*, 1993; Rohacek *et al.*, 2008). We have established that at the stage of sporophyte intensive growth this value was 0.610 for submerged and 0.778 for floating fronds that is an indirect evidence of incomplete formation of photosynthetic apparatus (Fig. 4). At the stage of sporophyte growth and sporocarps formation the value of potential quantum efficiency was 0.745 for submerged and 0.810 for floating fronds.

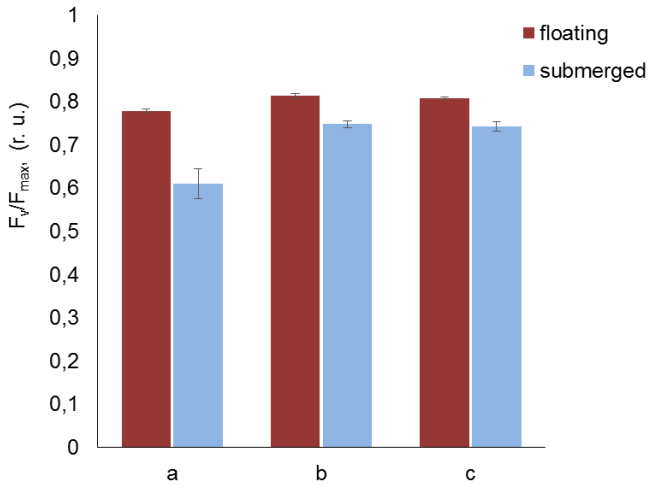

Fig. 4. Maximum potential quantum efficiency of photochemical reactions PSII in floating and submerged fronds of *S. natans* at different stages of ontogenesis: a – the first investigated stage, b – the second investigated stage, c – the third investigated stage

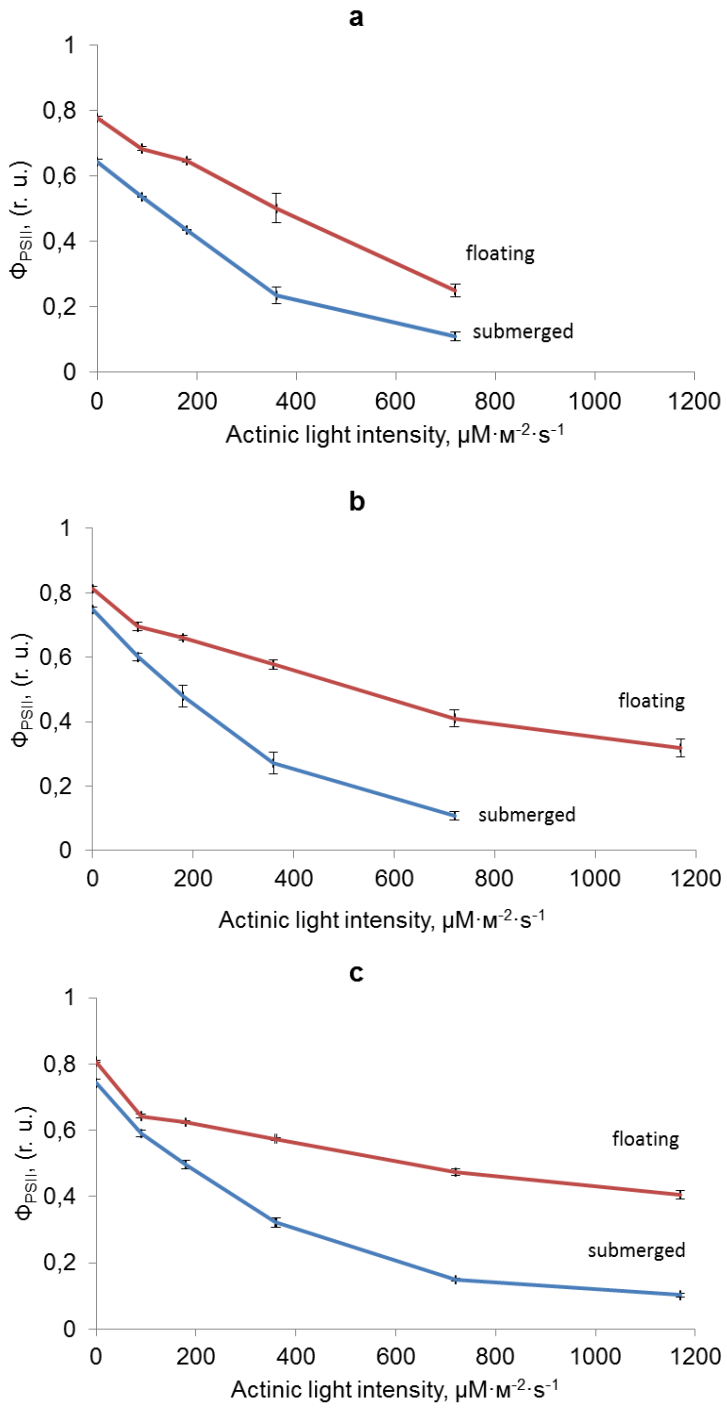

Fig. 5. Dependence of effective quantum yield (PSII) on actinic light intensity in floating and submerged fronds of *S. natans* at different stages of ontogenesis: a – the first investigated stage, b – the second investigated stage, c – the third investigated stage

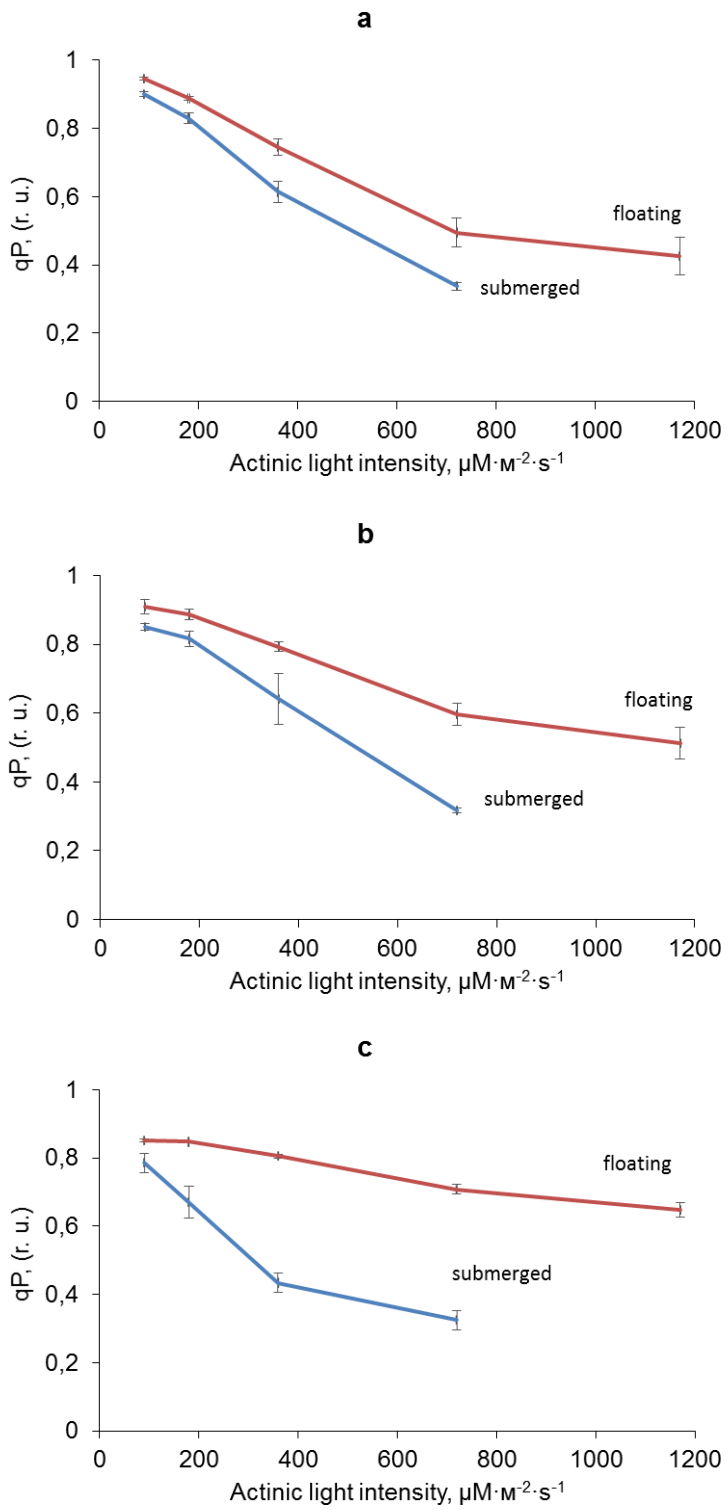

Fig. 6. Dependence of photochemical quenching (qP) on actinic light intensity in floating and submerged fronds of *S. natans* at different stages of ontogenesis: a – the first investigated stage, b – the second investigated stage, c – the third investigated stage

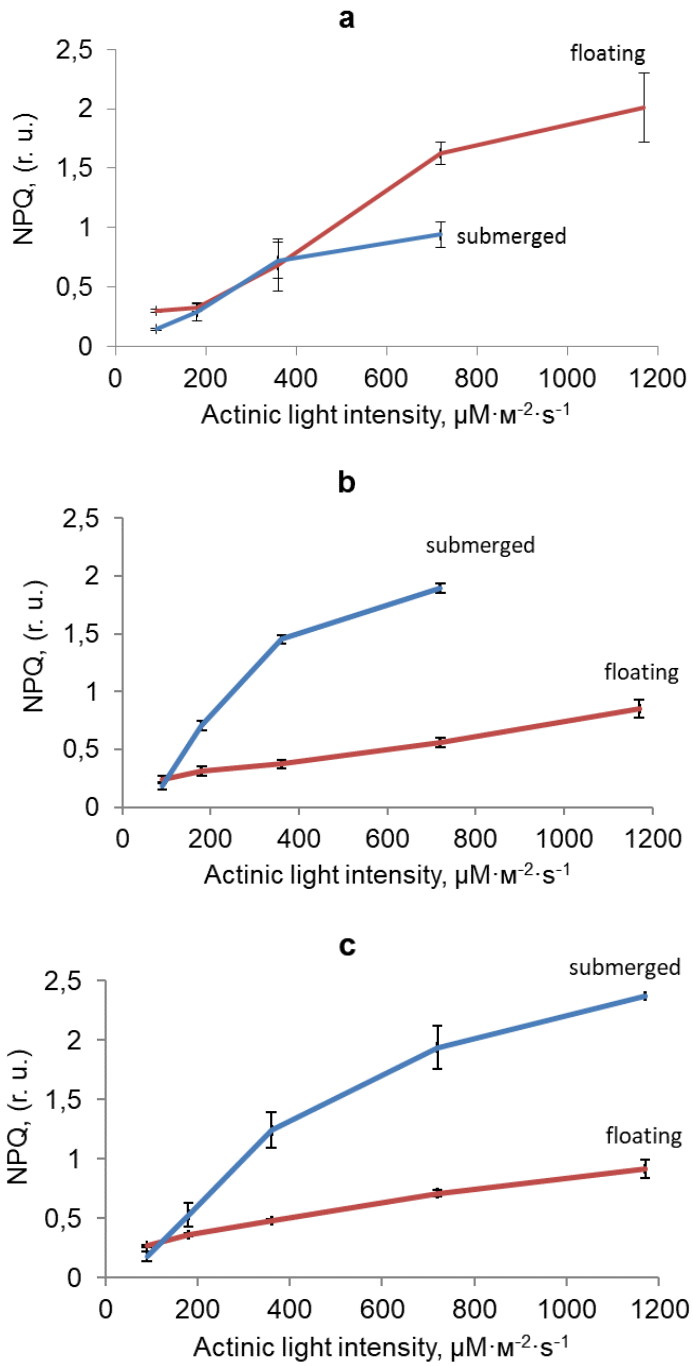

Fig. 7. Dependence of effective nonphotochemical quenching (NPQ) on actinic light intensity in floating and submerged fronds of *S. natans* at different stages of ontogenesis: a – the first investigated stage, b – the second investigated stage, c – the third investigated stage.

With different light intensities, values of the quantum efficiency PSII (Φ_{PSII}) and photochemical quenching (qP) were higher in floating fronds at all studied stages of sporophyte ontogenesis (Fig. 5, 6). It should be noted that floating fronds, even with the maximum light intensity of 1170 $\mu M \times m^{-2} \times s^{-1}$, had high values of Φ_{PSII} and qP, which amounted to 0.45 and 0.65, respectively. We think that the reason for that is a considerable adaptive ability of floating fronds, which under natural environment

conditions are quite often affected by light of intensity exceeding the above value. In submerged fronds the values of Φ_{PSII} and qP with light intensity of more than 200 $\mu M \times m^{-2} \times s^{-1}$ were much lower (Fig. 5, 6).

The obtained data indicate that utilization of light energy in photochemical reactions decreased and electron transport slowed down. Values of Φ_{PSII} and qP of floating fronds were found to increase at the final stages of sporophyte development (September) that is indicative of more effective reactions of the photosynthesis dark phase primarily, and of increased activity of RuBisCO, the key enzyme of the Calvin cycle. Values of the quantum efficiency and photochemical quenching of photosynthesis of submerged fronds were stably low throughout all studied stages of ontogenesis. In other authors` publications photoinhibition is associated with a low content RuBisCO in chloroplast stroma (Genty, 1989; Demming-Adams, 2008) that causes a physiological limit in the activity of dark photosynthetic reactions, which we detected in chloroplasts of submerged fronds.

Difference between fronds was more distinct for values of Φ_{PSII} than for those of qP. It is known that the qP value represents a transmission capacity of the photosynthetic electron-transport line and does not depend on non-photochemical energy quenching including damage of photosystems, while the Φ_{PSII} value inversely depends on these processes (Maxwell and Johnson 2000; Brestic and Zivcak, 2013). Thus, it may be suggested that the main reason of the difference we found in the Φ_{PSII} values of floating and submerged fronds is based on different levels of nonphotochemical energy quenching that consists of regulated quenching in antenna (active component) and photodamages to PSII (passive component). The value of nonphotochemical quenching NPQ represents some increase in the energy dissipation of absorbed light as heat radiation. This mechanism protects the reaction centers in conditions when light intensity exceeds the optimal level (Maxwell and Johnson, 2000). The measured NPQ values were within the range of 0.9-2.4 (Fig. 7) that is evidence of stable operation of the excess energy dissipation mechanism. In *S. natans* these values gradually increased along with increase of light intensity and reached the maximum at 1170 $\mu M \times m^{-2} \times s^{-1}$. In submerged fronds the measured NPQ values were much higher than in floating ones at the second and the third stages of ontogenesis, indicating low photosynthetic effectiveness and highly effective protective mechanisms. Thus, our studies have shown that floating fronds are characterized by an effectively operating photosynthetic electron-transport chain that promotes adaptation to intense lighting. Low photosynthetic effectiveness in submerged fronds under the maximum lighting is most probably conditioned by an excessive number of light-absorbing complexes that is required for an optimal light utilization for 24 hours or for shading other submerged photosynthetic organisms, which compete for resources (Melis, 2009).

4. Conclusion

Our study showed that chloroplast ultrastructural peculiarities, especially morphometric features of parenchyma cell chloroplasts at the stage of sporophyte intensive growth, indirectly indicate that the main producer of assimilates is the floating frond; at the stage of sporocarp formation partial chloroplast degradation occurs and a considerable number of plastoglobules emerge; the content of photosynthetic pigments in floating fronds is two times and at some stages three times higher than that of submerged ones; floating fronds are characterized by an effective operation of the

photosynthetic electron-transport chain and, correspondingly, by an efficient utilization of light energy of considerable intensity. Thus, the ability to effectively utilize light energy of increasing intensities, quantitative and qualitative composition of photosynthetic pigments, chloroplast ultrastructure, frond microstructure specificity contribute to a successful existence of the fern at the water-air boundary.

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