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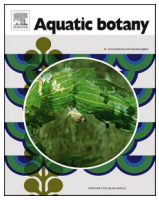

Understanding unique tolerance limits in *Hydrocotyle verticillata*: From submergence to water deficiency

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ABSTRACT

Hydrocotyle verticillata can tolerate varying degrees of flooding, up to complete submergence, and is at the same time extremely sensitive to drought. Understanding the structural and biochemical principles of these unusual tolerance limits is of particular importance. We analyzed the effect of soil flooding, complete submergence (rooted plants and floating stems), and dehydration on root anatomy, alcohol dehydrogenase (ADH), heat shock proteins 70 (HSP70), hydrogen peroxide, and DNA integrity using light microscopy, biochemical and histological methods. It was shown that anatomical traits of adventitious roots with a triarch stele were similar in the plants growing under different conditions; the single-layered and thin-walled epidermis formed relatively short root hairs; essential air spaces were absent in the cortex parenchyma. Results on ADH clearly showed that anaerobic energetic metabolism in root apices and individual rhizoderma cells of the mature root zone was normal for this species in optimal and suboptimal conditions, while leaves changed metabolism to anaerobic in response to submergence. Alterations in the protein spectrum were accompanied by adequate up-regulation of HSP70 under different levels of flooding and dehydration/rehydration. These results appear to indicate a flooding adaptation strategy for *H. verticillata* based primarily on metabolic plasticity rather than morphoanatomical adaptations. Most notably, the resistance of this species to long-term submergence has been associated with strong ADH induction in leaves, transient activation of 70 kDa isoform of HSP70 and induction of 66 kDa isoform, as well as a significant delay in the accumulation of hydrogen peroxide and DNA degradation.

1. Introduction

Hydrocotyle verticillata Thunb. (family Araliaceae) is native to North and South America, and distributed as an almost cosmopolitan species in flowing waters and shallow lakes in Asia and Australia (Winkel and Borum, 2009; Lim et al., 2014). It can be grown as an aerial-aquatic plant, as a terrestrial plant inhabiting wet soil areas, as well as fully submerged, rooted or floating, as a true aquatic plant. It is important to preserve biodiversity in the littoral zones, which is involved in biogeochemical processes in aquatic ecosystems (Bouchard et al., 2007). In addition, the invasive potential of *Hydrocotyle* species and their use for phytoremediation are also considered (Rejmánková, 1992; Hussner and Meyer, 2009; Lim et al., 2014).

H. verticillata is a perennial creeping plant with single leaves and filamentous roots in each node of a horizontal rhizome. Its umbrella-shaped leaves have a round bright green plate with a diameter of 0.5–6 cm and petioles up to 10 cm long. White bisexual flowers are gathered in whorls. Fruits are ovoid to ellipsoid, 1–3 mm wide, ribbed (Rejmánková, 1992; Seago et al., 2005; Winkel and Borum, 2009; Lim et al., 2014). This species is an ideal plant for paludariums, where it can grow on the border between aquatic and terrestrial areas, and is also widely cultivated as an attractive aquarium plant. In addition, it is considered a valuable medicinal plant containing a variety of biologically active chemical components that can be used as an adjuvant in various diseases (Lim et al., 2014).

Thus, the natural habitat of *H. verticillata* plants is floodplain areas, and they withstand the fluctuations in water levels in natural and artificial wetland ecosystems. However, we have little information on the cellular and molecular mechanisms of adaptation of this species to different water environments. Under flooding, the main stressful factor

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is known to be a limited supply of oxygen to the submerged parts of plants. Various structural, physiological, and metabolic changes occur in plants to adapt and survive in such conditions (Jackson and Ricard, 2003; Bailey-Serres and Voesenek, 2008; Colmer and Voesenek, 2009; Voesenek and Bailey-Serres, 2015). In particular, tolerance to oxygen deprivation upon flooding in many species is enhanced by aerenchyma, which provides internal gas movement from shoot to root. The critical role of energy metabolism in plant resistance to oxygen depletion has also been widely studied (Sachs and Vartapetian, 2007; Pan et al., 2021). Plants more tolerant to flooding have a more active anaerobic fermentation pathway which consists of two steps: carboxylation of pyruvate to acetaldehyde catalyzed by pyruvate decarboxylase (PDC) and the subsequent reduction of acetaldehyde to ethanol with concomitant oxidation of NAD(P)H to NAD(P)+ catalyzed by alcohol dehydrogenase (ADH) (Sachs and Vartapetian, 2007). *ADH1* expression is sensitive to waterlogging, takes part in plant adaptive processes in an anaerobic environment, and is necessary for plant survival under low oxygen conditions (Kyozuka et al., 1991; Chung and Ferl, 1999; Banti et al., 2008; Tougou et al., 2012; Xuan et al., 2021). Thus, the activity of ADH, which is a key enzyme in ethanol fermentation, is considered to be one of the important indices of plant resistance to oxygen shortage.

Key components of nonspecific cellular responses to variable environment are excessive production of reactive oxygen species (ROS) and induction of heat shock proteins (HSP), in particular under anaerobic conditions (Jackson and Ricard, 2003; Banti et al., 2008; Colmer and Voesenek, 2009; Chen et al., 2014; Gill et al., 2019) and water deficiency (Duan et al., 2010; Miller et al., 2010; Kordyum et al., 2019; Kozeko, 2021; Fu et al., 2023). ROS can cause oxidative damage to proteins, lipids, and nucleic acids (Juan et al., 2021), and, at the same time, are signaling molecules that enable cells to quickly react to various stimuli and activate stress response networks or induce programmed cell death (PCD) (Foyer and Noctor, 2005; Gechev et al., 2006; Miller et al., 2010; Mittler, 2017). In particular, hydrogen peroxide is one of the ROS types involved in the regulation of plant responses to various environmental stresses, in particular water deficiency or its excess. In turn, one of the early biochemical indicators of oxidative cell damage is a change in DNA integrity (Duan et al., 2010; Chen et al., 2014). HSPs, which function as molecular chaperones provide protection and restoration of protein homeostasis under stressful conditions (Feder and Hofmann, 1999; Sørensen et al., 2003; Liberek et al., 2008). Their up-regulation increases the resistance of plant organisms in variable environments. To investigate this aspect in *H. verticillata*, we chose the HSP70 family, inducible members of which are considered a major player of the stress response in many species (Sørensen et al., 2003; Kozeko, 2021).

Submergence tolerance of plants has been classified as the Low Oxygen Quiescence Syndrome (LOQS) and the Low Oxygen Escape Syndrome (LOES) (Bailey-Serres and Voesenek, 2008). The main features of LOQS plants are a non-elongated shoot, a high content of enzymes necessary for the formation of ATP under anaerobic conditions, economical use of ATP, and strengthening the mechanisms for protecting cells from the harmful effects of flooding. Traits of LOES plants are shoot elongation, and aerenchyma formation (Bailey-Serres and Voesenek, 2008; Colmer and Voesenek, 2009).

To elucidate the adaptive strategies that allow *H. verticillata* to live in environments with varying water levels, we conducted experiments simulating conditions from complete submergence to water deficiency and examined structural and molecular response including root anatomy and ADH as anaerobic adaptation traits, HSP70 and H_2O_2 as components of nonspecific stress response, as well as DNA integrity analysis in the experimental plants using by light microscopy, biochemical and histological methods.

2. Materials and methods

2.1. Experimental setup

Plants of *H. verticillata* were obtained from the collection of tropical plants in the M.M. Grishko National Botanical Garden of the National Academy of Sciences of Ukraine. Stem segments (clones) with a single nod from one plant were planted in pots with a diameter of 8 cm filled with well-moistened peat substrate (pH 5.5–6.5), with 5 replicates for each variant. The plants were grown as terrestrial plants at a soil water content (SWC) of 70–80 % fresh weight (FW), 22–24 °C, 16 h light/8 h dark and light intensity 100 μmol m^{-2} s^{-1}. After one month, when the plants had 10–15 leaves, they were subjected to different water conditions: (1) terrestrial (control); (2) soil flooding as aerial-aquatic plants; (3) complete submergence; (4) stem segments with nods immersed in water as floating plants; and (5) dehydration/rehydration, when terrestrial plants were subjected to progressive soil drying after watering ceased until leaf wilting, followed by rehydration for 2 h (Fig. 1).

2.2. Analysis of root anatomy

Root anatomy was studied in terrestrial (control) plants and plants after 10 days of soil flooding, submergence and floating. To obtain sections of the root mature zone, root tips of 1.5 cm long were fixed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) under vacuum for 4 h, then fixed with 1 % OsO_4 in the same buffer at 4 °C for 4 h. Dehydration and embedding in an epon-araldit mixture was done following Carde (1987). Semi-thin sections (1.0–1.5 μm) were cut on ultramicrotome MT-XL (RMC-Boeckeler Instruments, USA), stained with 1 % toluidine blue in 1 % sodium borate. Sections were examined using Olympus BX53 light microscope (Olympus Corporation, Japan); photomicrographs were taken using Olympus LC30 digital camera and Olympus cellSensEntry software. Root diameter, central cylinder diameter and cortex thickness were measured on digital images of cross-sections of the root mature zone in ten biological samples with three measurements in each, using the ImageJ software (https://imagej.nih.gov).

2.3. Analysis of alcohol dehydrogenase

To analyze alcohol dehydrogenase (alcohol:NAD oxidoreductase, ADH, EC 1.1.1.1) in leaves, terrestrial (control) plants and plants after 1 day of soil flooding, 1 and 10 days of submergence, and dehydration were used. Native electrophoresis and ADH staining in PAG were performed. 0.3 g of leaf plate was ground in a mortar with liquid nitrogen, then 0.5 ml of extraction buffer containing 0.1 M Tris-HCl (pH 7.0), 10 % glycerol, 0.5 % dithiothreitol, 1 % Triton X100 was added. The homogenate was centrifuged for 5 min at 5 000 rpm and 4 °C. An equal quantity of total protein of each sample was separated by native electrophoresis in 6 % polyacrylamide gel (PAG). Incubation of a gel with a solution containing 10 mM NAD, 10 mM nitroblue tetrazolium (NBT), 10 mM phenazine methosulphate (PMS), and 0.6 % ethanol in 1 M Tris-HCl buffer (pH 8.0) was performed to detect enzymatic activity in PAG. Three biological replicates were conducted.

The localization of ADH in roots was determined in terrestrial (control) and floating plants according to the method of Porterfield et al. (1997) with modifications. Root tips up to 2 cm long were incubated in tubes with a solution containing 3 mM $MgCl_2$, 1 % sucrose, 0.6 mM NBT, 1 mM PMS, 0.5 mM NAD, 2 % polyvinylpyrrolidone, 0.01 % Tween 20 in 20 μM cacodylate buffer (pH 7.4) with 0.5 mM ethanol. ADH staining was first performed in the dark at 40 °C for 60 min as described by Porterfield et al. (1997). Next, to more clearly characterize the differences between root growth zones, incubation was carried out at room temperature for 5–40 min. In addition, ethanol-free staining of the enzyme was performed to determine its activity that can be achieved through the use of an endogenous substrate. Part of the roots in each

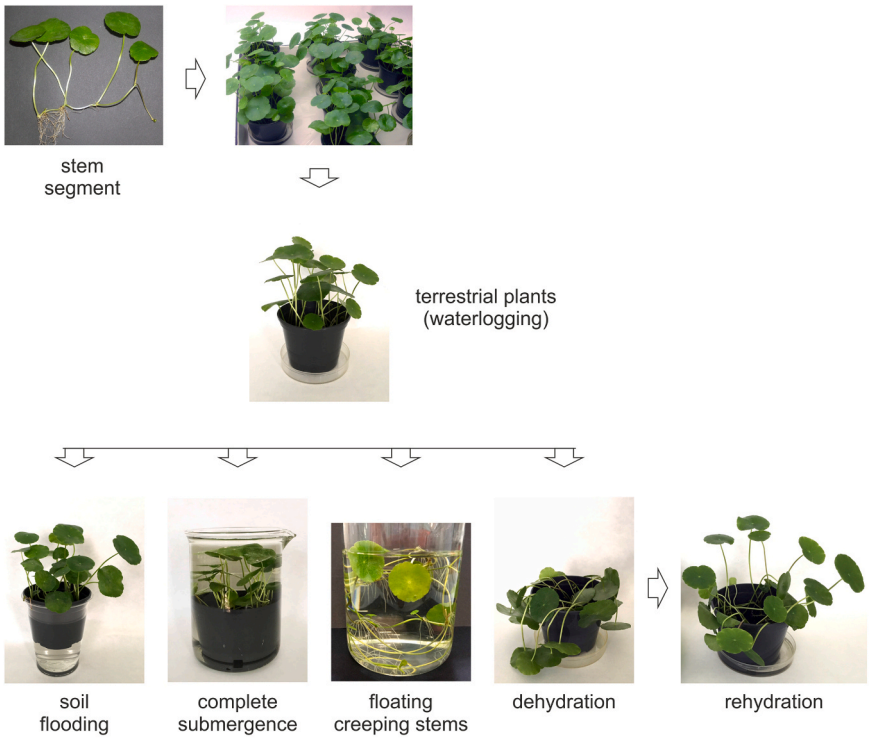

Fig. 1. Scheme of experiments with *Hydrocotyle verticillata* under various conditions of water supply, ranging from water deficiency to complete submergence.

variant was also subjected to triple freezing (−20 °C) – thawing to remove endogenous ethanol before staining and check the specificity of the histochemical reaction according to Baxter-Burrell et al. (2003). After staining roots were photographed. Five to ten biological replicates were conducted.

2.4. Protein extraction and Western-blot analysis

Leaves of terrestrial (control) plants and plants after 1 day of soil flooding, 1 and 10 days of submergence, and dehydration/rehydration were used for protein analysis. Soluble proteins were extracted following the method described by Krishna and Kanelakis (2003). 0.3 g of leaf plate was ground in a mortar with liquid nitrogen, then extraction buffer containing 25 mM Tris-HCl (pH 8.0), 20 mM NaCl, 1 mM EDTA, 1 mM protease inhibitor phenylmethylsulfonyl fluoride was added. The homogenate was centrifuged for 15 min at 12,000*g* and 4 °C. Part of the supernatant was used to determine the protein concentration according to the method of Bradford (1976). SDS-buffer contained 0.125 M Tris-HCl (pH 6.8), 4 % SDS, 20 % glycerol, and 5 % β-mercaptoethanol was added to aliquots of the supernatant in a 1:1 ratio. Electrophoretic separation of protein was performed in vertical 10 % PAG under denaturing conditions (Laemmli, 1970). After electrophoresis, gels were either stained with Coomassie G-250 or used for immunoblotting. Western-blotting was performed as described earlier (Kozeko et al., 2015). The molecular weight of proteins was determined using markers of 10–180 kDa (TermoFisher) and GelAnalyzer 2019.1 software (http://www.gelanalyzer.com/). Three biological replicates were conducted.

2.5. Histochemical detection of hydrogen peroxide

Detection of H_2O_2 production in leaves and roots was conducted based on the method described by Gill et al. (2019). Terrestrial (control) plants and plants subjected to soil flooding for 10 days, submergence for 1, 5 and 10 days, and dehydration were used for analysis. Detached leaf plates and root tips 1.5 cm long were immersed in 1 mg/ml 3, 3′-diaminobenzidine (DAB) (pH 3.8) and incubated at 25 °C in the dark. The roots were stained for 1–2 h and rinsed once with 2-N-morpholino-ethanesulfonic acid/potassium chloride (Mes/KCl) buffer (3–10 M, pH 6.15). The leaves were stained for 5 h and then cleared from chlorophyll in boiling ethanol (96 %) for 10 min. The stained samples were photographed·H_2O_2 accumulation in the tissues was estimated as a red-brown polymerization product. Five biological replicates were conducted.

2.6. DNA isolation and electrophoresis

Analysis of DNA integrity was conducted in submerged plants. Green leaves were collected after 1, 2, 3, 4, 5, 10 and 30 days of submergence. Total DNA was isolated from leaf blade tissues by the CTAB method as described by Weigel and Glazebrook (2002), with some modifications. 100 mg of leaf blades were ground in a microtube with 500 µl of CTAB buffer (2 % CTAB, 1.4 M NaCl, 20 MM EDTA, 100 MM Tris-HCl, pH 8.0) and the homogenate was incubated at 65 °C for 60 min. DNA was extracted with chloroform/isoamyl alcohol (24:1) and precipitated with isopropanol for 10 min. Pellet was washed with 70 % ethanol, dissolved in TE buffer (10 MM Tris-HCl, pH 8.0, 1 MM EDTA) and treated with RNase A to degrade residual RNA. To visualize DNA fragmentation, 3 µg of DNA of each sample was separated by electrophoresis on 2 % agarose gel containing ethidium bromide at 5 V/cm for 1.5 h (Duan et al., 2010). Two biological replicates were conducted.

2.7. Statistical analysis

The quantitative data are presented as mean (M) ± standard deviation (SD) of three independent experiments. For statistical analysis of the significance of differences between the variants, a Student's *t*-test was used ($p < 0.05$).

3. Results

3.1. Plant response to various water conditions

In this study, the effect of various water supply conditions on *H. verticillata* plants (clones) was examined. Plants grown as (1) terrestrial (control) were subjected to (2) soil flooding, (3) complete submergence, (4) floating stem segments, (5) dehydration/rehydration (Fig. 1). It should be noted that because *H. verticillata* is moisture-loving, the terrestrial plants were grown in soil with high water content (70–80 % SWC) that was close to water-saturated soil (i.e. waterlogging). Observations during a month of experiment showed that plants growing under soil flooding did not differ phenotypically from terrestrial ones. In submerged plants, the apical growth of the stem was delayed, the leaf blades gradually darkened, and 17.5 ± 5.2 % of the leaves died within a month. On floating stem segments, numerous adventitious roots emerged from the nodes on the first day of submersion, which within 10 days reached a length of 1–3 cm, and then their growth was delayed. In the case of dehydration, gradual drying of the soil from 70.0 ± 3.5 % to 47.0 ± 1.6 % SWC decreased the water content in the leaves from 86.9 ± 0.05 % to 83.6 ± 0.03 % of FW resulting in a loss of leaf turgor. After rehydration for 2 h, the water content in the leaves increased to 86.5 ± 3.2 %, and the turgor of most of the leaves was restored.

3.2. Root anatomy

To assess the changes in root anatomy and formation of aerenchyma in flooded roots, transverse and longitudinal sections of the mature zone were examined. It was found that the anatomy of the nodal adventitious roots in plants growing at different flooding levels was similar to that in terrestrial plants, which is shown in two contrasting variants – terrestrial and floating plants (Fig. 2A and Fig. A2, data on flooded and submerged plants not shown). Slender roots had a triarch central cylinder or stele: three xylem discrete strands alternate with the phloem located near the periphery of the stele. Thin-walled epidermal cells were flattened and long, certain of them formed rather short hairs in the mature zone of adventitious roots being both in the soil (waterlogging, soil flooding, complete submergence) or in the water (floating stems). Parenchymatous cortex cells varied in size and shape. Large air spaces were absent in the cortex. The innermost layer of the cortex with Casparian strips, endodermis and pericycle were clearly visualized on sections. When comparing terrestrial and floating plants, smaller root diameter and central cylinder diameter with the same cortex thickness were determined in floating plants (Fig. 2B).

3.3. Alcohol dehydrogenase synthesis in leaves and roots

ADH in leaves was determined using native electrophoresis of proteins followed by staining of the enzymatic reaction product in the gel. The presence of ADH in roots was revealed by histochemical staining.

ADH zymograms of leaves in all variants contained 2 bands with the enzyme activity in the gel (Fig. 3). The content of the more mobile isoform was stable under various experimental conditions, while the synthesis of the less mobile one varied significantly. In particular, soil flooding did not cause a noticeable activation of the synthesis of the latter in the leaves. Significant induction of this isoform was detected in leaves after 1 day of complete submergence, and its content remained high after 10 days. In addition, a small amount of both ADH isoforms was also detected under water deficiency.

Determination of ADH in roots was initially carried out for two contrasting options - terrestrial and floating plants. Histochemical staining with ethanol in the staining solution at 40 °C for 1 h gave a dark blue coloration of the *H. verticillata* root tips, which was dense in floating plants and variable (dense, gradient, discrete) in terrestrial plants (Fig. A1). A staining pattern similar to that of terrestrial plants was also observed in roots in flooded soil (data not shown). To reveal differences between the root growth zones in floating plants, staining was carried out under milder conditions, at room temperature for 5–40 min.

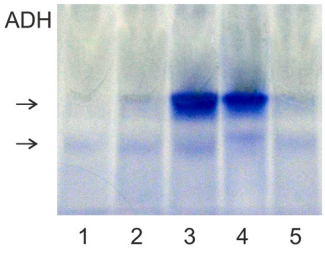

Fig. 3. Expression of ADH in leaves of *H. verticillata* under (1) terrestrial conditions (control), (2) soil flooding for 1 day, (3) submergence for 1 day, (4) submergence for 10 days and (5) loss of the leaf turgor under water deficiency. Native gel electrophoresis of the protein followed by staining for ADH activity was performed.

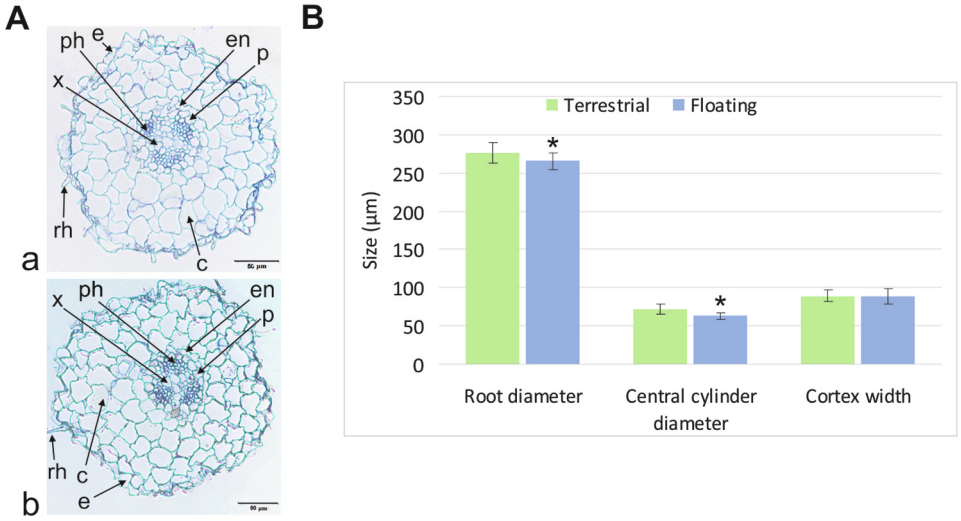

Fig. 2. Root anatomy of terrestrial and floating *H. verticillata* plants. (A) Root cross sections of (a) terrestrial (control) and (b) floating plants. Abbreviations: e – epidermis, c – cortex, en – endodermis, p – pericycle, ph – phloem, x –xylem, rh - root hair. (B) Root diameter, central cylinder diameter, and cortex thickness of the root mature zone. Data are M ± SD of 10 replicates. Means followed by asterisks indicate significant differences (Student's *t*-test, $p < 0.05$).

Staining for 5 min showed the highest enzyme activity in the root meristem zone of most roots (Fig. 4). Increasing the duration of staining clearly showed the presence of ADH also in the elongation zone and in the individual rhizoderma cells of the mature zone (separately shown in the version with 40-minute staining, Fig. 4). The latter can be characterized as mosaicism. Root incubation in the staining solution without ethanol revealed ADH activity in the same root zones, but the stain intensity was noticeably weaker compared to the roots stained with exogenous ethanol (Fig. 5).

3.4. Protein spectrum and HSP70 level in leaves

Electrophoretic separation in SDS-PAG of soluble leaf protein showed noticeable changes in protein composition depending on the experimental conditions (Fig. 6A). The maximum changes affected the ~50 kDa protein zone, which, in terms of molecular weight and high content, can be attributed to the large subunit of ribulose bisphosphate carboxylase (Rubisco, a key enzyme of the Calvin cycle) (Andersson, 1996; Liese et al., 2023). Its amount increased in leaves after 1 day of soil flooding and complete submergence, but drastically decreased during prolonged submergence. Determination of HSP70 by Western blotting revealed two protein bands of 70 kDa and 66 kDa (Fig. 6B). 70 kDa protein was present in terrestrial plants, and its synthesis activated after 1 day of soil flooding and especially after 1 day of submergence. In contrast, content of this HSP70 significantly decreased after 10 days of submergence, while 66 kDa isoform was induced. Under water deficiency, rapid loss of the leaf turgor under soil drying to ~47 % SWC was accompanied by a sharp decrease in the amount of the 50 kDa protein band (presumably Rubisco) and an increase in HSP70 (70 kDa) level. After 2-h rehydration, a substantial level of HSP70 and a rapid recovery of a high content of 50 kDa protein were determined in the leaves restored turgor, but amount of both proteins was low in the leaves, which remained wilted and subsequently died.

3.5. Hydrogen peroxide production in leaves and roots

Histochemical staining with 3,3′-diaminobenzidine (DAB) was used to assess changes in H_2O_2 production in roots and leaves under different water conditions. The reaction product of a characteristic red-brown

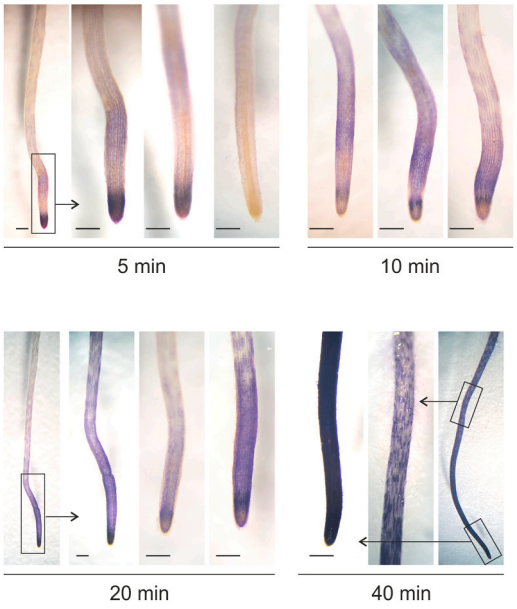

Fig. 4. Histochemical detection of ADH enzymatic activity in root apices of floating *H. verticillata* plants incubated in ADH stain solution for 5–40 min. Bar – 0.3 mm.

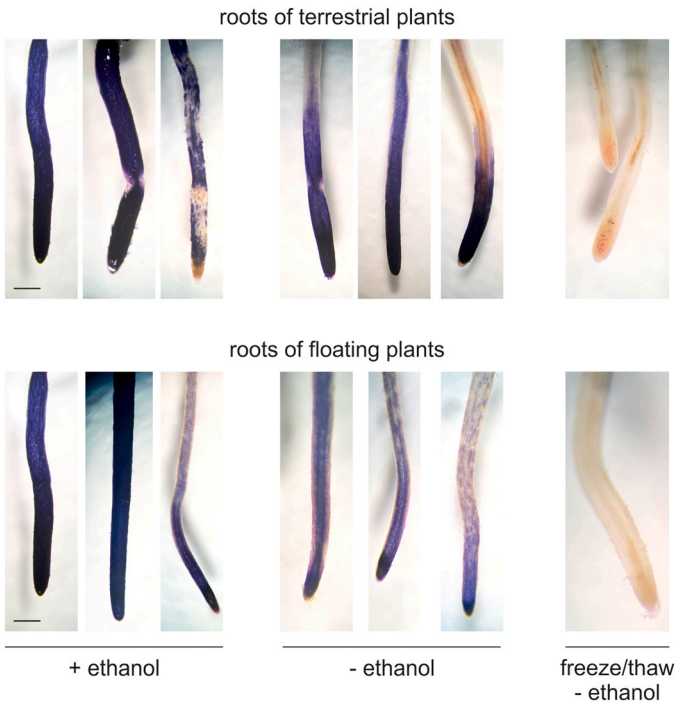

Fig. 5. Histochemical detection of ADH enzymatic activity in root apices of terrestrial (control) and floating *H. verticillata* plants incubated in ADH stain solution for 40 min with and without ethanol, as well as with freeze-thaw treatment (to remove the endogenous substrate). Bar – 0.3 mm.

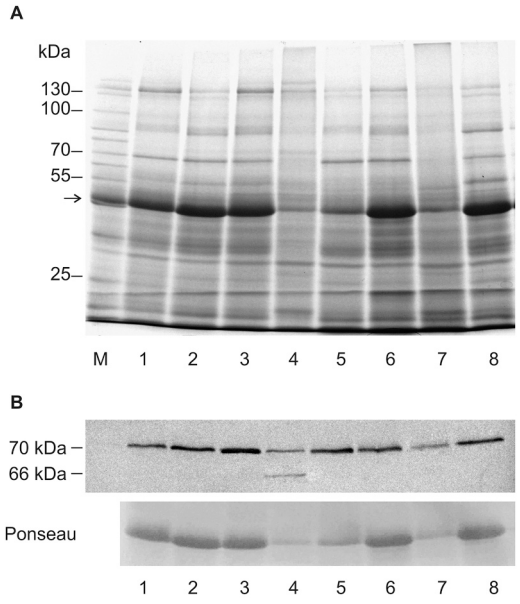

Fig. 6. Electrophoretic protein pattern (A) and HSP70 (B) in leaves of *H. verticillata* plants under different water levels: (1) terrestrial (control) plants, (2) soil flooding for 1 day, (3) submergence for 1 day, (4) submergence for 10 days, (5) loss of the leaf turgor under water deficiency and (6, 7) after 2-h rehydration in the leaves, which restored turgor (6), and in the leaves, which remained wilted (7), (8) terrestrial plants subjected to 40°C for 1 h (internal control for HSP70 induction). (A) Electrophoretic pattern of soluble proteins in 10 % SDS-PAG. (M) Molecular weights (kDa) of marker proteins. The arrow points to the protein zone of ~50 kDa (presumably the large subunit of Rubisco). (B) Western blot of HSP70. Ponceau staining was applied as a control.

color was revealed along the root with the highest intensity in the apical meristem and elongation zone under different experimental conditions (Fig. 7B). A less intense staining in roots was found during soil flooding, and the most intense staining was observed under water deficiency. In leaves, the product of DAB staining was not detected in terrestrial plants, as well as under soil flooding (Fig. 7A). In leaves of submerged plants, H_2O_2 production appeared after 5 days and reached the significant level after 10 days. Under dehydration, a red-brown product was detected along the edges of leaves that had lost turgor. In leaves unable to restore turgor during rehydration, tissue death began from this zone (Fig. A3).

3.6. Evaluation of DNA integrity in submerged leaves

Given that the highest level of H_2O_2 production in leaves was revealed in submerged plants, which can lead to DNA fragmentation, DNA integrity analysis in green leaves of these plants was performed. As shown in Fig. 8, weak DNA smearing appeared following 2 days of submergence and slightly increased over 10 days. Extensive DNA degradation was detected after 30 days of submergence. DNA 'laddering' into nucleosome fragments was not revealed.

4. Discussion

4.1. Alcohol dehydrogenase response to different water conditions

The results of the investigation convincingly showed that *H. verticillata* is very sensitive to water deficiency and simultaneously highly resistant to flooding, up to complete submergence. The cultivation of this moisture-loving species as terrestrial plants was carried out in soil with high water content that brought these conditions closer to waterlogging with excess water in the root zone. Such soils are usually O_2-deficient, which leads to root hypoxia. Considering the ways of adaptation of this species to oxygen shortage, the following should be noted. Slender roots of dicotyledonous *H. verticillata* are morphologically similar to the roots of monocotyledonous aquatic plants, e.g. *Lemna* and *Spirodela* species (Huang et al., 2018). However, we did not observe large air spaces nor lacunae in the cortex, although lacuna onset in the

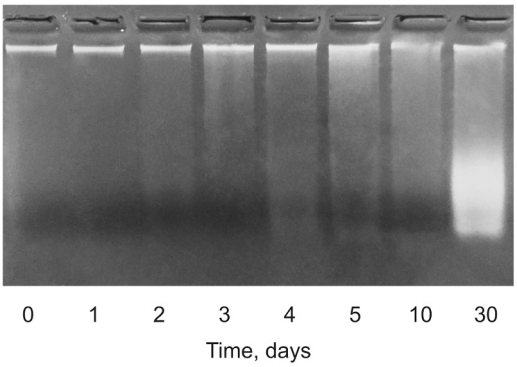

Fig. 8. Dynamics of DNA destruction in leaves of *H. verticillata* plants during submergence for up to 30 days. DNA was isolated from the leaves of control and treated plants and analyzed by agarose gel electrophoresis.

apical meristem of *H. verticillata* roots was described earlier (Seago et al., 2005). Thus, our data showed that the roots of this species did not form either constitutive or flood-induced aerenchyma characteristic of many wetland species (Seago et al., 2005; Colmer and Voesenek, 2009). On the other hand, a high level of ADH activity in the root tips was observed both in terrestrial *H. verticillata* plants and under all types of flooding, indicating the function of ethanol fermentation in the roots under optimal and suboptimal conditions. This, in turn, confirms the literature data that roots exposed to hypoxia are characterized by high activity of anaerobic metabolism (Porterfield et al., 1997; Jackson and Ricard, 2003; Sachs and Vartapetian, 2007). At the same time, we did not observe ADH response in leaves exposed to with air, even when plants were subjected to soil flooding, but it was strongly induced in leaves experiencing submergence. This situation is somewhat different from those in many other species, when ADH was not detected in the roots of plants grown at 60–70 % SWC and quickly appeared in the root tips and temporarily in the leaves after soil flooding, for example, in *Glycine max* (Tougou et al., 2012), *Cucumis sativus* (Xu et al., 2014), *Gossypium hirsutum* (Zhang et al., 2016), *Sium sisaroideum* (Kozeko and Ovcharenko,

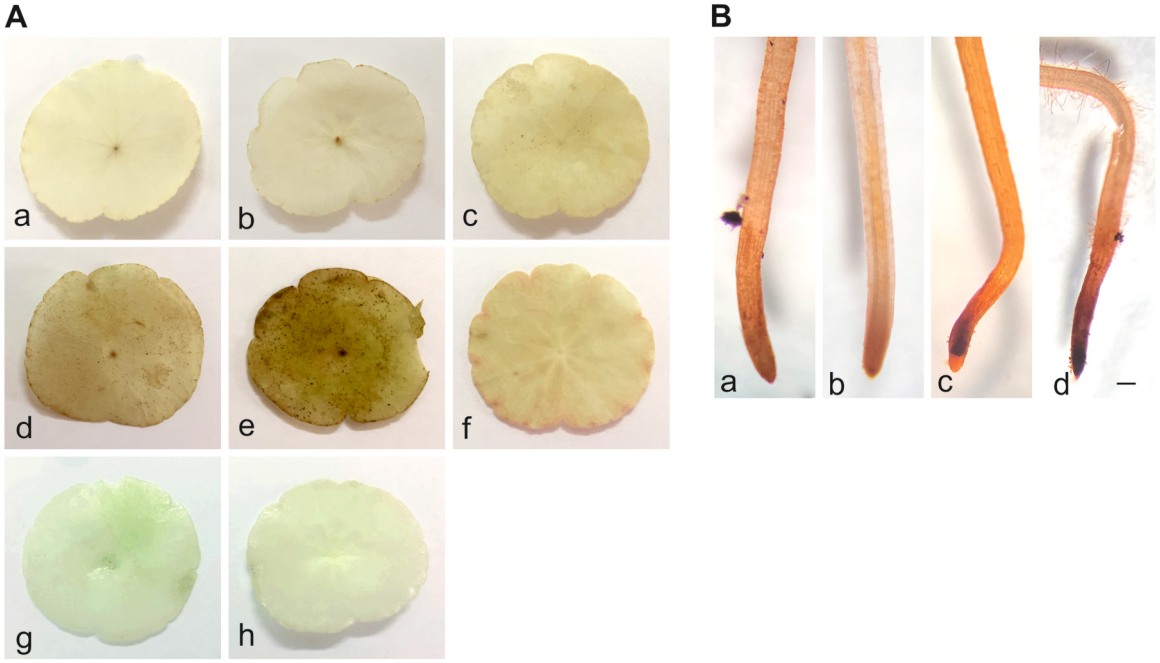

Fig. 7. Histochemical detection of H_2O_2 production in *H. verticillate*. (A) Leaves under (a) terrestrial (control) conditions, (b) soil flooding for 10 days, (c, d, e) submergence for 1, 5 and 10 days respectively, (f) loss of the leaf turgor under water deficiency, and (g, h) control without DAB staining for terrestrial and submerged plants respectively. (B) Roots under (a) terrestrial (control) conditions, (b) soil flooding for 10 days, (c) floating and (d) water deficiency; bar – 0.2 mm.

2015; Kordyum et al., 2017), *Hordeum vulgare* (De Castro et al., 2022). In this case, the synthesis of ADH in response to soil flooding began in the roots and then appeared in the leaves, which can be explained by the transmission of the hypoxia signal from the roots to the above-ground parts of the plants and, thus, a systemic reaction (Chung and Ferl, 1999; Kozeko and Ovcharenko, 2015). Obviously, the absence of such reaction dynamics in *H. verticillata* plants may be explained by their adaptation to high soil moisture (~80 % SWC), apparently close to optimal for this species. It is noteworthy that a small amount of ADH was also found under dehydration, which may indicate a certain contribution of alcoholic fermentation to the energy metabolism of this species under water shortage.

The high ADH activity inherent in plants growing in natural waterlogging conditions enables them to withstand flooding. For example, this has been demonstrated in two populations of the Central Amazon tree *Himatanthus sucuuba.* Seedlings from the floodplain population showed that high ADH activity during the entire 4-month flood ensured their survival, while its activity in seedlings from the upland population peaked about 15 days after submersion followed by a continuous decrease and death of all the plants (Ferreira et al., 2009). Seedlings of the evergreen perennial shrub *Distylium chinense* distributed in China, Indonesia, India, Japan, Korea and Malaysia, especially in the riparian areas and wetlands of the Yangtze River in China, showed high ADH activity in roots and leaves during submergence and its gradual decrease after soil drainage (Sun et al., 2020). According to the authors, the ability of this species to survive during long-term submergence is provided by a gradual transition from mitochondrial aerobic respiration to ATP generation via glycolysis and fermentation. In our opinion, a significant prolonged induction of ADH synthesis in the leaves and maintenance of its high level in the roots of submerged *H. verticillata* plants is an important adaptive mechanism that allows them to survive long-term submergence.

It should be noted that in situ ADH staining in *H. verticillata* roots revealed the highest activity of the enzyme in the meristem and elongation zones. This is generally consistent with the localization of *ADH1* promoter activity in maize (Kyozuka et al., 1991), indicating that the tissue-specific pattern of ADH synthesis is determined by its promoter activity. In flood-sensitive soybean seedlings, anaerobic conditions caused an increase in both ADH mRNA and protein in the root tips (Tougou et al., 2012). The localization of the maximum ADH activity in the root tips indicates that meristematic and elongating cells are the most sensitive to oxygen supply due to the need for high energy production during division and growth. Remarkably, ADH staining without exogenous ethanol showed the same spatial pattern of enzymatic activity, but of lower intensity. This difference may indicate that the synthesis of ADH in the root cells is carried out with some excess. The mosaic pattern of the color reaction product in the root rhizoderm of *H. verticillata* might be associated with the presence in this layer of two types of cells that form and do not form root hairs. It is known that the cells that form root hairs are distinguished by a special type of metabolism. However, the nature of this phenomenon requires further research.

4.2. Changes in the protein spectrum and HSP70 level

Changes in the electrophoretic protein spectrum of *H. verticillata* leaves reflect alterations in protein homeostasis in response to various water supply conditions, and also indirectly indicate reprogramming of gene expression. The most quantitative changes were noted for the ~50 kDa protein band, which presumably attributed to the large subunit of Rubisco, a key enzyme of photosynthesis and photorespiration (Andersson, 1996; Liese et al., 2023). Its degradation is known to be a common reaction of plant cells to various stressful impacts. An increase in its amount both after 1-day soil/complete flooding and during rehydration after water deficiency approved the need for this moisture-loving plant in conditions of high water supply for intensive photosynthesis. Noteworthy, high levels of this protein were restored upon rehydration in leaves that restored turgor, but kept low in leaves, which remained wilted and subsequently died. On the other hand, a strong decrease in the level of this protein during both prolonged submergence and loss of turgor under water deficiency is apparently associated with a decrease in photosynthetic activity. Both drought and flooding are well known to adversely affect the photosynthetic system and its efficiency (Hussner and Meyer, 2009; Chen et al., 2014; Machado et al., 2023).

As mentioned above, HSPs play a key role in protecting protein homeostasis, especially during reprogramming protein synthesis in response to environmental influences (Feder and Hofmann, 1999; Sørensen et al., 2003; Liberek et al., 2008). An increase in the HSP70 level in *H. verticillata* was determined in response to flooding and dehydration. Activation of 70 kDa isoform synthesis in leaves during soil flooding indicated a systemic stress reaction to root hypoxia. This is consistent with the results of our previous study of *Sium sisaroideum* and two *Malva* species, where root flooding caused progressive activation of HSP70 synthesis in the leaves (Kozeko and Ovcharenko, 2015; Kozeko and Rakhmetov, 2016). A significant transient increase in the level of 70 kDa isoform in leaves after 1 day of submergence, as well as the induction of 66 kDa isoform after 10 days reflected the dynamics of HSP70 system response to severe hypoxic conditions in this species. The elevated amount of 70 kDa isoform in leaves that lost and then restored turgor during dehydration/rehydration (while the content of Rubisco changed significantly), as well as its low level in leaves that remained wilted, showed the importance of this chaperone for survival of *H. verticillata* under water shortage. HSP70 induction was earlier determined in slowly drained rice (Wang et al., 2011) and in macroalgae *Agarophyton vermiculophyllum* during desiccation and rehydration (Fu et al., 2023). It is noteworthy that the activation of HSP70 synthesis in wilted leaves of *H. verticillata* was determined at ~47 % SWC, which indicates its high sensitivity to dehydration. For comparison, up-regulation of HSP70 in two *Malva* species was shown at ~25 % SWC (Kozeko and Rakhmetov, 2016), in *Agrostis* sp. – at ~7 % SWC (Li et al., 2018).

In general, these results are consistent with the literature data describing transcriptional and translational reprogramming in response to energy deprivation (Baena-González and Sheen, 2008; Branco-Price et al., 2005) and water deficiency (Bray, 2002). In particular, significant alterations in the transcriptome and differential translation of cellular mRNAs were shown in *A. thaliana* upon hypoxia including preferential translation of HSP- and ADH-encoding mRNAs (Branco-Price et al., 2005).

4.3. Hydrogen peroxide production in roots and leaves under different water conditions

Both flooding/hypoxia and drought can lead to increased ROS production in tissues, which, in turn, can cause oxidative stress and alterations in the protein spectrum (Colmer a Voesenek, 2009; Miller et al., 2010). In *H. verticillata*, H_2O_2 was determined in roots, mainly in their tips, in all variants, most of all under water deficiency, which corresponds to their high sensitivity to water shortage. In leaves, H_2O_2 was not detected upon soil flooding, but its active production was revealed after 5 days of complete submergence and reached a significant level after 10 days, herewith the leaves remained viable. In leaves, that lost turgor in case of water deficiency, more H_2O_2 amount was noted at the edge of the leaf plate, which showed accumulation of ROS in the peripheral leaf region, from which tissue death began.

These findings are generally consistent with the literature data, which describe active production of ROS, including hydrogen peroxide, in different species both under water deficiency (Duan et al., 2010; Kordyum et al., 2019; Fu et al., 2023) and flooding (Chen et al., 2014; Gill et al., 2019). For example, aerial-aquatic plants adapted to oxygen deprivation have to adjust to an eventual decrease in the soil water

content under natural conditions (Ferreira et al., 2009). Water deficiency has been reported to cause more ROS production in terrestrial plants compared to aerial-aquatic plants, such as *Alisma plantago-aquatica* and *Sium sisaroideum*, closely related to aerial-aquatic *S. latifolium* (Kordyum et al., 2019). The ROS accumulation in macroalgae *Agarophyton vermiculophyllum* during desiccation was shown to match the expression of the respiratory burst oxidase homologs (Rboh) gene that indicates ROS as a signal of desiccation stress (Fu et al., 2023). A higher level of ROS production was reported to be characteristic of *Zea mays* hybrids less resistant to hypoxia (Gill et al., 2019). Nevertheless, it should be emphasized that, in contrast to the less moisture-loving species, H_2O_2 was not detected in *H. verticillata* leaves during soil flooding and slowly accumulated upon submergence.

Accumulation of ROS in cells can cause direct DNA destruction or trigger internucleosome fragmentation of DNA as a part of PCD (Duan et al., 2010). For example, soil flooding for 3–4 days led to progressive H_2O_2 production, DNA 'laddering' and PCD in maize leaves (Chen et al., 2014). Unlike this, our results showed that complete submergence of *H. verticillata* plants caused only slight DNA smearing in leaves for at least the first 10 days, while strong DNA degradation (without DNA 'laddering') was detected after 30 days, which may be associated with leaf senescence. This ability of cells to preserve DNA integrity under submergence for a long period of time may also partly explain the high flood resistance of this species to submerged conditions. Such slow degradation of DNA might be associated with high antioxidant activity in the cells, which requires further research. It is known that H_2O_2 can modulate the expression of numerous genes, including those encoding antioxidant enzymes (Gechev et al., 2006; Wan et al., 2009; Mittler, 2017). Increased activity and/or quantities of components of the antioxidant system, in turn, are associated with plant resistance to various adverse factors. Given the above, we speculate that increased H_2O_2 production in *H. verticillata* plants could be considered an important "oxidative signal", which enhances water stress tolerance by modulating the expression of tolerance-associated genes (Foyer and Noctor, 2005; Mittler, 2017) that allows plants to grow and develop under moderate water fluctuations, as well as "oxidative stress" leading to plant death under strong and prolong stressful impacts.

Collectively, the obtained results provide insight into the nature of the tolerance limits of *H. verticillata*, which is very sensitive to water deficiency and tolerant to hypoxia at various levels of flooding. Anaerobic energetic metabolism in root apices and individual rhizoderma cells of the mature root zone is normal for this species, while leaves change metabolism to anaerobic in response to submergence, which indicates their metabolic plasticity. In addition, adequate dose-dependent H_2O_2 production and HSP70 synthesis is involved in cellular response to both water excess and shortage. Submerged *H. verticillata* plants were characterized by delay of shoot growth, absence of developed aerenchyma in roots, high level of ADH necessary to generate some ATP without O_2, and increased production of HSP70 that counteract the disruption of protein homeostasis caused by flooding. Taken together, all this makes it possible to classify this species as a LOQS plant (Bailey-Serres and Voesenek, 2008; Colmer and Voesenek, 2009).

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CRediT authorship contribution statement

L. Kozeko: Conceptualization, Species identification, Methodology, Investigation, Data analysis, Figures, Writing. **Y. Ovcharenko:** Investigation, Data analysis, Figures. **S. Jurkonienė:** Formal analysis, Writing – review & editing. **E. Kordyum:** Funding acquisition, Conceptualization, Methodology, Formal analysis, Writing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors declare no conflict of interest.

Data Availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aquabot.2023.103725.

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