Effect of hypoxia and post-hypoxia on the fluctuations in contents of malate and citrate, the activity of malic enzyme, and on the intensity of gas exchange in moss gametophores

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Abstract

Mosses are plants of simple anatomical structure and as they occur in habitats characterised not only by major changes in the concentrations of carbon dioxide, they suffer the stress of periodic water shortages or submergence in water. The condition of hypoxia (submergence in water or $CaCl_2$ solution) prompted the increase in daily fluctuations in malate content, particularly in the gametophores of *Polytrichum piliferum* Hedw. No significant increases in daily fluctuations of citrate were found in the hypoxia and post-hypoxia conditions. Placing gametophores for 168 h in air with a concentration of CO_2 at ~ 350 μmol mol^{-1}, and 21% of oxygen, after being submerged for 24 h in water, reduced the daily fluctuations of malate and citrate. Keeping the plants in these conditions for a long time (120–168 h) produced the increase in photosynthesis intensity in the gametophores of *Mnium undulatum* Hedw. and *P. piliferum* by 13% and 51%, respectively, when compared with plants submerged for 24 h. The intensity of respiration during post-hypoxia, however, was markedly lower compared with the intensity of the process recorded in hypoxia, particularly in the gametophores of *P. piliferum*. The increased daily fluctuations of malate and NAD(P)H in the studied species under hypoxia could constitute an important element of adaptive strategy to these conditions.

Additional key words: calcium; hypoxia; malate; *Mnium*; photosynthesis; *Polytrichum*; post-hypoxia; respiration.

Introduction

Malate, citrate, and NAD(P)-ME play essential roles in a number of important metabolic processes, contributing to the proper course of photosynthesis and respiration. The limited access to oxygen and also anoxia are common environmental challenges faced by plants. Snow and ice melting, spring floods, and excessive rains are among the natural conditions leading to hypoxia (1–5 kPa) or anoxia (0 kPa) (He *et al*. 1996, Felle 2005). Some aquatic plants develop structural and metabolic adaptations in order to adjust to the oxygen deficit (reducing synthesis, accumulation of energy, changes in pH of cytoplasm, anaerobic fermentation, increased Ca^{2+} ion concentration in cytosol, and changes in the functioning of the cell membrane, *etc*.) (Davies 1986, Kronzucker *et al.* 1998, Bacanamwo and Purcell 1999, Igamberdiev and Hill 2004).

Damage to green plants during inundation in water is associated with reduced access, not only to oxygen, but also to carbon dioxide dissolved in water. The decreased oxygen concentrations in the aquatic environment is associated with the slow rate of diffusion of this gas in water and its limited solubility (Xia and Roberts 1994, Mohanty and Ong 2003). In the roots of plants exposed to hypoxia, increased activities of phospho*enol*pyruvate carboxylase were noted (Edwards *et al.* 1998, Sairam *et al.* 2008). In these plants, the rate of malate synthesis by PEPC is much higher than the rate of its metabolism by malic enzyme (ME). This demonstrates the fact that the activity of this enzyme is not limited by the reserves of malate *in vivo.* Additionally, ME shows high activity after only a few minutes of hypoxia (Edwards *et al.* 1998, Lemaire *et al.* 2005).

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Abbreviations: CAM – Crassulacean acid metabolism; GOT − glutamate oxaloacetate transaminase (EC 2.14.1.1); ME − malic enzyme; NAD-ME − NAD-malic enzyme (EC 1.1.1.38); NADP-ME − NADP-malic enzyme (EC 1.1.1.40); PEPC – phospho*enol*pyruvate carboxylase (EC 4.1.1.31); P_N − net photosynthetic rate; R − respiration; ROS – reactive oxygen species; SOD – superoxide dismutase (EC 1.15.1.1).

The stress conditions favour the production of reactive forms of oxygen and thus increase the photooxidative damage. The oxidative stress results from a number of environmental factors (Blokhina *et al.* 2003, Rzepka *et al.* 2005). It may occur in the conditions of limited availability of oxygen, which can be divided into three different physiological states: hypoxia, anoxia, and reoxygenation. The production of a reactive oxygen species (ROS) is characteristic of hypoxia, and particularly of reoxygenation. ROS emerge in the reactions catalysed by various enzymes, such as: lipoxygenases, peroxidases, and oxidases. Most susceptible to the damage caused by free radicals are lipids (peroxidation of unsaturated fatty acids in membranes), proteins (denaturation), carbohydrates, and nucleic acids. The consequences of hypoxia caused by oxidative stress depend on the type of tissue, species of plant (tolerance to anoxia), the properties of the membrane, the content of the endogenous antioxidant, the ability to induce a response in the antioxidative system, *etc*. (Thomas et *al.* 2005, Chen *et al*. 2007). The accumulation of ROS is prevented by the system of antioxidants, namely: compounds of low molecular weight (ascorbic acid, glutathione, tocopherols), enzymes regeneration *via* reduced forms of antioxidants, and enzymes such as, for example, SOD, peroxidases and catalases (Votrubova *et al.* 1992).

Calcium ions are an important factor integrating the cellular response to various stress factors, and their concentrations in cells increase under the effect of stress factors. The shortage of oxygen (hypoxia) may trigger oxidation stress. The accumulation of H_2O_2 and the products of lipid peroxidation indicate that in an environment with a low level of oxygen, the production of ROS increases. Changed concentrations of calcium ions in the cytosol provide a signal to change a number of metabolic processes, the expression of genes associated with the stress of oxygen shortages in the environment (He *et al.* 1996, Rzepka and Krupa 2008).

The malate and citrate changes are accompanied by either an uptake or release of CO_2. The availability of such compounds as malate, citrate, and pyruvate change with alterations in light/dark conditions, and depends also on the intensity of photosynthesis and respiration. Furthermore, all C_4 enzymes and metabolites occur in C_3 plants, although they are less active and accumulate in different tissues. In C_4 plants, the NADP-ME catalyses the oxidative decarboxylation of L-malate, so producing pyruvate, CO_2, and NAD(P)H (Lai *et al.* 2002). An isoform of this type of enzyme occurs in the cytosol and plastids, and NADP-ME isoforms are also found in the chloroplasts of bundle sheath cells of C_4 plants, as well as in the cytosol of some CAM plants (Haslam *et al.* 2003, Rut *et al.* 2008).

Mosses are organisms that differ greatly from higher plants. They are the oldest plants, in evolutionary terms, and were amongst the first to colonise a terrestrial environment. The two moss species employed in the experiments differ in terms of leaf structure and environmental requirements. *M. undulatum* occupies habitats with increased moisture levels, whereas *P. piliferum* is a moss growing on sands in open spaces (Rzepka *et al.* 2005).

The objective of this study was to check whether the increase in malate and citrate contents occurs in these plants under the conditions of hypoxia and post-hypoxia. Additionally, an attempt to connect the fluctuations in malate content with the changes in NAD(P)-ME activity was made. As either an uptake or release of CO_2 is associated with the metabolism of malate and citrate, the intensity of the gas exchange was determined. In order to ascertain, whether the changes in malate contents are associated with CO_2 availability, quantities of this compound were determined in plants with limited concentrations of this gas. The submergence of plants in water not only restricts their access to oxygen but also alters the nature of the carbon source (CO_2, HCO_3^-, CO_3^{2-}).

Materials and methods

Plant material: The gametophores of two moss species, *M. undulatum* and *P. piliferum*, were collected from a natural environment and grown in a chamber with the photoperiod set for 12 h (07:00−19:00) of light and 12 h (19:00−07:00) of darkness. The irradiance was 80 µmol m^{-2} s^{-1} PAR, the temperature 15±2°C, and the relative air humidity was ~ 95%.

Stress factors: The plants were subjected to hypoxia stress through submergence in water for 24 h (12/12 h). During that time, the irradiance and temperature conditions were identical to those in the growing chamber. The gametophores were then placed for 168 h in an atmosphere, where the concentration of CO_2 was ~ 350 µmol mol^{-1}, and that of oxygen 21%. The mosses were also kept for 24 h in CO_2-free water; in CO_2-free air; and also in a $CaCl_2$ solution with a concentration of Ca^{2+} ions at 0.9 mM. After the prescribed period under conditions of hypoxia or post-hypoxia, the contents of malate and citrate, the activity of ME, as well as the intensity of the gas exchange, were determined.

Determination of malate and citrate contents: A certain quantity of plant material (0.10 – 0.15 g of leaves gametophores) was ground with distilled water in a ratio of 1:4 (w/w) at 4°C, and then centrifuged *(MPW-6, MPW-Med*, Warsaw, Poland) for 5 min at 5,000 × *g* at the same temperature. The malate content in plants was determined towards the end of the day (19:00) and again near the end of the period of darkness (07:00). The malate and citrate contents were calculated per 1 g of the fresh mass (FM) and expressed as the difference in contents:

Δ malate [μM] = $(\text{malate})_{\text{night}} - (\text{malate})_{\text{day}}$ according to Möllering's method (1985).

The content was determined in a spectrophotometer (*Aquarius, CE 9500, CECIL*, Cambridge, England) on a wavelength at λ = 340 nm, at room temperature. The sample contained: 500 μl of buffer (0.6 M glycylglycine, 0.1 M L-glutamic acid of pH 10), 100 μl NAD^+, 5 μl GOT, 725 μl redistilled H_2O, 25 μl of the examined sample, and 5 μl of L-malic dehydrogenase.

Δ citrate [μM] = $(\text{citrate})_{\text{night}} - (\text{citrate})_{\text{day}}$ according to Möllering's method (1985).

The sample contained: 180 μl of buffer (0.5 M glycylglycine, 0.6 mM $ZnCl_2$ of pH 7.8), 25 μl NADH, 10 μl MDH/LDH, 331 μl redistilled H_2O, 25 μl of the examined sample, and 5 μl of citrate lyase.

Determination of NADP-ME and NAD-ME activity: The activity of the enzyme was determined by the method described by Ashton *et al*. (1990). Prior to the measurements, the plant material was kept in the same conditions as during the malate and citrate contents determinations. The activities of NADP-ME and NAD-ME in the studied plants were measured near the end of the light period (19:00) and near the end of the dark period (07:00). The contents of NADPH or NADH were calculated in μM per 1 g of FM.

NADP-ME: Plant leaves were homogenised with the addition of 2 cm^3 of a buffer (pH 7.5) containing 50 mM HEPES, 5 mM dithiothreitol (DTT), and 5 mM EDTA. The buffer was then increased to a volume of 5 ml. The homogenate was then transferred to Eppendorf tubes and centrifuged for 5 min at 5,000 × *g*. Then, 0.2 cm^3 of a pH 8.3 buffer containing: 25 mM Tricine, 0.1 mM EDTA, 2 mM $MgCl_2$, 5 mM L-(-)-malic acid, and 0.5 mM $NADP^+$ were added to 0.1 cm^3 of supernatant. The samples were then incubated in 25ºC for 1 h. The amount of released NADPH was assayed spectrophotometrically at 340 nm. The activity of the enzyme can be found on a calibration curve prepared by measuring standards with concentrations from 0 to 5 μM NADPH.

NAD-ME: Plant leaves were homogenised with the addition of a 2 cm^3 pH 7.5 buffer containing: 50 mM HEPES, 5 mM DTT, 0.25% PVP-40 (polyvinylpyrrolidone), 0.5% Triton, and 2 mM $MnCl_2$. The buffer was increased to a volume of 5 ml. Homogenate was then transferred to Eppendorf tubes and centrifuged for 5 min at 5,000 × *g*. Next, 0.2 cm^3 of buffer (pH 8.3) containing: 25 mM HEPES, 0.2 mM EDTA, 2 mM $MnCl_2$, 5 mM L-(-)-malic acid, 5 mM DTT and 2m M NAD, was added to 0.1 cm^3 of the supernatant. After ~ 10 min, 0.1 cm^3 of the 0.4 mM solution of coenzyme A was added.

Gas exchange measurements: The net photosynthetic rate (P_N) and the rate of respiration (R) in the studied mosses were determined with an *ADC-225 MK-3* infrared gas analyser (*ADC BioScientific Ltd*., Herts, England). The irradiance reaching the plants during the photosynthesis measurements was 100 μmol m^{-2} s^{-1} PAR. A stable temperature of 25ºC was maintained throughout all photosynthesis and respiration measurements. The intensities of these processes were determined in an atmosphere containing 21% of oxygen, whereas the concentration of CO_2 was ~ 350 μmol mol^{-1} in a closed system. The total volume of the entire system was 1.74 dm^3, and air was passed through a washer with distilled water, in order to maintain RH at 70%. The intensities of the processes were expressed as percentages of the initial values (prior to submergence in water).

Results

The moss species employed in this study differ daily in terms of their fluctuations in malate and citrate content (Rzepka *et al.* 2009). The differences in malate and citrate content between day and night were small, when the gametophores of *M. undulatum* and *P. piliferum* were kept in a CO_2-free atmosphere (Table 1). Such results may signify a reduced level of phospho*enol*pyruvate carboxylase. When the studied gametophores were placed under conditions of limited access to oxygen (hypoxia) by submerging them in water with a normal CO_2 content, the differences in daily fluctuations of malate in the gametophores of *P. piliferum* amounted to ~ 8 mmol kg^{-1}(FM), whereas in the leaves of *M. undulatum,* the increase in Δ malate was slight. The difference in malate content between day and night was slight. When the plants were submerged in CO_2-free water, the difference in the malate content during day and night in the gametophores of *M. undulatum* and *P. piliferum* was much smaller compared with the control plants (Table 1).

A significant difference in malate content (Δ) was found in the leaves of *P. piliferum* and *M. undulatum,* submerged in a solution containing 0.9 mM Ca^{2+} and amounted to ~ 10 mmol kg^{-1}(FM). Mosses submerged for 24 h in this solution showed a marked increase in malate content both after period of light and of darkness (Table 1).

The increase in the difference in malate content is caused by a 24-h submergence in water of the gametophores of *M. undulatum* and *P. piliferum,* whereas after moving them to air containing 21% oxygen, a gradual decline occurs in the difference between contents of this compound (Fig. 1*A*).

The changes in the activity of ME are associated with malate content. The activity of NADP-ME in the post-hypoxia period is partly correlated with the changes in malate content. After a temporary increase in the activity of this enzyme, a gradual decrease in its activity occurred during 7 days of post-hypoxia (Fig. 1*C*). The activity of NAD-ME under conditions of hypoxia and post-hypoxia

Table 1. Changes in malate, citrate, NADPH, and NADH contents [mmol kg^{-1}(FM)] in the gametophores of *M. undulatum* and *P. piliferum,* kept for 24 h under various concentrations of carbon dioxide. Means ± SD, $n = 4$.

		control			air – 0% CO_2			submergence in water			submergence in water 0% CO_2			submergence in water + 0.9 mM Ca^{2+}		
		light	dark	Δ	light	dark	Δ	light	dark	Δ	light	dark	Δ	light	dark	Δ
Mnium undulatum	malate	19.2 ±0.21	30.9 ±0.26	11.7 ±0.31	14.9 ±2.25	21.4 ±0.70	6.5 ±1.62	20.8 ±2.69	35.0 ±4.91	14.2 ±2.32	12.7 ±3.31	16.9 ±2.63	4.2 ±0.61	18.4 ±2.65	38.6 ±4.31	10.2 ±1.32
	citrate	12.0 ±0.17	14.2 ±0.10	2.2 ±0.18	10.6 ±1.12	12.3 ±0.91	1.7 ±0.20	11.1 ±2.97	13.8 ±3.61	2.7 ±5.21	9.7 ±1.93	12.3 ±2.42	2.6 ±0.64	11.5 ±2.20	15.2 ±1.35	3.7 ±0.32
	NADPH	2.09 ±0.25	2.32 ±0.20	0.23 ±0.11	1.98 ±0.51	2.11 ±0.36	0.13 ±0.06	2.06 ±0.09	2.53 ±0.09	0.47 ±0.01	2.02 ±0.80	2.23 ±1.01	0.21 ±0.13	2.21 ±0.31	2.54 ±0.30	0.33 ±0.11
	NADH	0.83 ±0.06	0.99 ±0.04	0.16 ±0.02	0.89 ±0.13	0.99 ±0.21	0.10 ±0.03	1.14 ±0.16	1.36 ±0.06	0.22 ±0.01	1.02 ±0.21	1.14 ±0.06	0.12 ±0.11	0.75 ±0.08	0.92 ±0.03	0.17 ±0.07
Polytrichum piliferum	malate	20.5 ±0.35	24.5 ±0.31	4.0 ±0.10	17.2 ±0.90	20.5 ±1.25	3.3 ±0.31	17.9 ±3.34	26.0 ±2.46	8.1 ±1.56	16.9 ±1.33	18.4 ±2.30	1.5 ±0.31	21.0 ±2.93	30.7 ±4.07	9.7 ±1.37
	citrate	10.0 ±0.15	12.6 ±0.14	2.6 ±0.10	9.2 ±0.32	11.0 ±0.71	1.8 ±0.22	11.2 ±2.81	14.9 ±1.96	3.7 ±1.04	10.2 ±1.13	11.8 ±1.61	1.6 ±0.34	9.2 ±2.14	13.5 ±0.91	4.3 ±0.61
	NADPH	1.13 ±0.18	1.2 9±0.21	0.16 ±0.04	1.02 ±0.23	1.09 ±0.08	0.07 ±0.01	0.82 ±0.04	1.12 ±0.03	0.30 ±0.01	1.08 ±0.11	1.20 ±0.21	0.12 ±0.07	1.05 ±0.11	1.22 ±0.17	0.17 ±0.05
	NADH	0.97 ±0.13	1.14 ±0.18	0.17 ±0.08	0.83 ±0.08	0.94 ±0.06	0.11 ±0.03	0.96 ±0.08	1.16 ±0.01	0.20 ±0.07	0.92 ±0.06	1.08 ±0.21	0.16 ±0.04	0.91 ±0.09	1.13 ±0.21	0.22 ±0.08

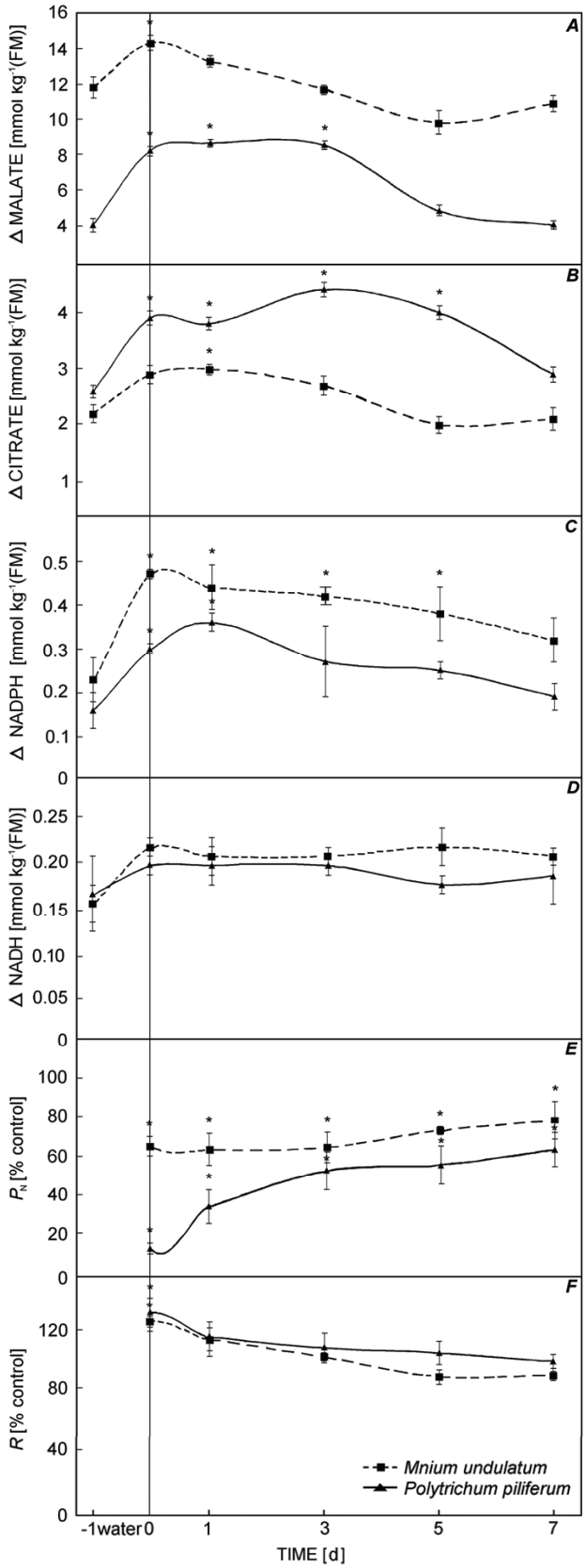

Fig. 1. Changes in *A*: malate, *B*: citrate, *C*: NADPH, and *D*: NADH contents, intensity of *E*: photosynthesis (P_N), and *F*: respiration (R) in the gametophores of *M. undulatum* and *P. piliferum,* during 24-h submergence in water and during the post-hypoxia period. * above bars indicates significance of differences from control plants at $p<0.05$. Means ± SD, $n = 5$.

is low, and there is only a slight increase in the gametophores of *M. undulatum* (Fig. 1*D*).

The differences between the day and night citrate content in the leaves of *M. undulatum* and *P. piliferum* are very small (Table 1). The changes noted over 24-h periods in the citrate contents, in plants kept under stress conditions, are much smaller than those in the malate concentrations. The fluctuations in citrate content in plants remaining in the state of hypoxia increase markedly in *P. piliferum*, whereas the oscillations of the contents of this compound in the leaves of *M. undulatum* are close to the control values (Table 1). The fluctuations in citrate content in submergence conditions in solutions containing calcium ions are distinct in the leaves of *P. piliferum* and *M. undulatum* and amount to 4.3 and 3.7 mmol kg^{-1}(FM). In the studied gametophores of *M. undulatum* and *P. piliferum,* the change in citrate content is only slight as in the case of submergence in CO_2-free water (Table 1). Submerging the gametophores of *M. undulatum* and *P. piliferum* for 24 h brings about a slight change in the citrate content, whereas after moving mosses to air with 21% of oxygen, the difference in the content of this compound occurs, particularly towards the end of the period of post-hypoxia (Fig. 1*B*).

The activity of the NADP-ME is higher in the leaves of *M. undulatum* than in *P. piliferum,* signified by the changes in NADPH content after the light and dark periods (Table 1). However, the activity of the NAD-ME isoform is low in both studied species. Under stress conditions, the activity of the NADP-ME isoform undergoes much greater changes than NAD-ME. The quantity of NADPH produced under hypoxia in leaves, as a result of the activity of the malate enzyme, increases markedly in the leaves of *M. undulatum* and *P. piliferum*. No significant changes were found, however, in the content of NADH in plants kept under these conditions. When submergence is made in a water solution containing calcium ions, the NADP-ME activity increases markedly in the *M. undulatum* leaves, whereas, the increase in the activity of this enzyme in *P. piliferum* is only slight. In these conditions, the changes in NAD-ME activity are slightly significant. Keeping plants in carbon dioxide free air or in CO_2-free water, reduces both the activity of NADP-ME and NAD-ME (Table 1).

The changes in P_N in the gametophores of *M. undulatum* and *P. piliferum* during 24-h submergence in water are also significant. The intensity of the process after 24-h hypoxia decreases in the gametophores of *M. undulatum* and *P. piliferum* by 35% and 88%, respectively

(Fig. 1*E*). Moving them to an atmosphere containing 21% of oxygen, results in an increase of photosynthesis in the gametophores of *M. undulatum* and *P. piliferum* by 13% and 51%, respectively, compared with the intensity of the process after 24-h hypoxia. The water-submergence stress causes a drop in photosynthetic activity, coupled with a simultaneous increase in the intensity of respiration, which increases by ~ 25–30%. After moving mosses to air containing 21% of oxygen, a gradual decline in the intensity of this process occurs, and near the end of the period they are kept in air, its intensity approaches its initial value (Fig. 1*F*).

Discussion

The accumulation of malate and, to some extent, of citrate could be an adaptation of the species being studied, to adverse conditions associated with hypoxia and post-hypoxia. Similarly, the change in the activity of NAD(P)-ME may result from the inclusion of this enzyme into the plant's response to a given stress factor, *i.e*. submergence in water. The increase of malate contents in the gametophores of submerged mosses may stem from changes in the activities of key enzymes associated with the metabolism of this compound, *i.e*. pyruvate dehydrogenase and phospho*enol*pyruvate carboxylase. The increase in the activity of these enzymes is characteristic for hypoxia conditions (Subbaiah and Sachs 2003, Marques da Silva and Arrabaca 2004). In aquatic plants grown under low CO_2 concentrations, the accumulation of malate increases at the expense of intermediate compounds involved in the Calvin cycle. Moreover, under these conditions, the value of CO_2 compensation concentration drops, whereas the activities of PEPC and ME increase (van Ginkel *et al*. 2001). The changes in NAD(P)-ME activity and the increase in malate concentration in the moss gametophores examined could result from similar changes in the metabolism as those occurring in aquatic plants. The lack in the activity of NAD-ME in stress conditions may confirm the lack of any significant role played by this enzyme in the response to these stress factors by plants under study. The intensity of photosynthesis in submerged gametophores shows a decrease after a short period in water, whereas respiration does not change substantially. In stress conditions, malate may also serve as an additional source of carbon, at the same time providing the means to accumulate carbon dioxide (Heber *et al.* 2001, Crecelius *et al.* 2003). It may be supposed that this increased quantity of malate in the studied moss species was associated with the photosynthetic carboxylation of CO_2. The decrease in malate concentration in plants kept in CO_2-free air or water suggests, however, that this compound could provide a source of carbon after CO_2 is released by the ME.

The photosynthetic activity in the studied gametophores is strongly inhibited in the conditions of hypoxia caused by total submergence in water. However, the presence of calcium ions in the solution results in reducing the effects of hypoxia on the intensity of photosynthesis in the mosses. The magnitude of changes in the intensity of photosynthetic assimilation of CO_2 under conditions of hypoxia in *P. piliferum* and *M. undulatum* is different. The changes in Ca^{2+} ion concentration in the cell may play an essential role in the reaction of plants to stress conditions. The concentration of calcium ions in the cell affects the conformation of protein structures. The activation of Ca^{2+}-binding proteins is one of the stages in information transfer, involving the stimulation of protein kinases. The hypoxia conditions may induce changes in the concentrations of calcium ions in the cytoplasm, which may in turn provide an important intracellular regulator of physiological and biochemical processes (Virolainen *et al*. 2002, Rzepka and Krupa 2008). The transportation of these ions across cell membranes affects the Ca^{2+} levels in the cytosol. This transportation may lead to concentration differences in cellular structures which, in the case of calcium ions, may constitute an element in the transduction path of the information on *e.g*. an action by a given stress factor. The increase in calcium ion concentration in cytosol could be one of the symptoms of oxidation stress (Virolainen *et al*. 2002, Zhao and Tan 2005). In the moss species under study, the calcium ions may play a role of information transducer and provoke the expression of genes associated with the stress resulting from an oxygen shortage in the environment.

In the natural environment, *P. piliferum* grows in conditions of relatively high irradiation (Rzepka *et al*. 2009). Also, it may not be excluded that in post-hypoxia, the oxidation stress may emerge in this moss species. The accumulation of malate during the night may be one of the adaptations to the post-hypoxia conditions. When facing a lack of oxygen, a continuous production of antioxidants is not always associated with increased antioxidation protection. The effectiveness of the protection depends on the location, the level of synthesis and transport of antioxidants, and the ability of various antioxidant systems to activate one another (Heber *et al*. 2001, Zhao and Tan 2005).

The changes in malate and citrate contents, as well as in the activity of ME, affect not only the gas exchange but also pH. The proposed biochemical model of intercellular regulation of pH involves a full set of carboxylating enzymes with overlapping optima in the physiological range of pH. The model proposed by Davies (1986) involves cooperation between ME and PEPC. In acidic pH, ME at this range of reactions being in its optimum increases the activity decarboxylating malate, which results in shifting pH towards alkaline range. Alternatively, when alkalisation occurs in cytoplasm, PEPC which has its optimum in this range,

elevates its activity. The latter results in increased concentration of oxaloacetate which is converted to malate by malate dehydrogenase in an reversible reaction. Malate contributes to a change in pH and acidification follows. In some plants, the synthesis of malate occurs at too slow pace to be useful in pH regulation. Nevertheless, this fundamental principle of biochemical regulation of pH is universally accepted (Davies 1986, Edwards *et al.* 1998).

The results obtained may suggest that increased amounts of malate and citrate, as well as increased activity of NADP-ME in the studied plant species, in conditions of hypoxia and post-hypoxia, could be an important element of their strategy to adapt to these conditions, so enabling these species to achieve great flexibility in terms of changes in the metabolism (gas exchange, pH).

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