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BRIEF COMMUNICATION

Effect of abiotic stress factors on fluctuations in contents of malate and citrate and on malic enzyme activity in moss gametophores

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Abstract

Diurnal fluctuations in the contents of malate in gametophores of *Polytrichum commune* Hedw. and *Polytrichum piliferum* Hedw. were small. In gametophores of *Mnium undulatum* Hedw. and leaves of *Hieracium pilosella* L. significant differences were found in the accumulation of malate between day and night. However, no significant diurnal differences were found in the contents of citrate. High irradiance, desiccation, and submergence by water resulted in increases in daily fluctuations of malate, particularly in the gametophores of *P. piliferum* and leaves of *H. pilosella*. Accumulation of malate during night may show the adaptation of the studied species to unfavourable conditions caused by stresses. The change in activity of NADP-malic enzyme may characterize a response to stress factors.

Additional key words: *Hieracium*; *Mnium*; NAD- and NADP-malic enzymes; *Polytrichum*; phospho*enol*pyruvate carboxylase; pyruvate orthophosphate dikinase.

The biosynthesis of organic compounds, transformation of compounds (photosynthesis, respiration), and mineral metabolism are connected with malate. This compound is an intermediate link participating in the transformation processes of saccharides in mitochondria. For these organelles, malate is an important substrate, because a significant portion of the process of glycolysis participates in the Krebs cycle, as it is connected with the activity of phospho*enol*pyruvate carboxylase (PEPC), malate dehydrogenase, and malic enzyme (ME) (Crecelius *et al.* 2003). The dynamic transformations of malate in leaf cells are essential to the osmotic status of the cell, turgor, and the diurnal changes in pH. Although the cell malate homeostasis depends on the accumulation of the compound in vacuoles, the changes in its content occur in a day/night cycle, and also throughout the growth and development of a plant (Lüttge 2002). The extent of the differences probably reflects the demand for this compound during the changes in cell activity. The compound which is transported either in a reduced form or as a negative anion is important to the course of the processes of photosynthesis, respiration, glyoxysomal/peroxisomal activity, and nitrogen assimilation.

Under stresses, the energy production/consumption is usually upset because the processes in the dark phase of photosynthesis are more delayed than the absorption of energy and the transport of electrons. Malate is significant for the coordination of these processes (Edwards *et al.* 1998, Chen *et al.* 2002). Stresses support the generation of reactive oxygen species (ROS) and increase photooxidation damage (Heber *et al.* 2001).

The oxidative decarboxylation of malate is a process typical of plants. The reaction results from the activity of a specific enzyme, namely ME, whose activity is found in all plant mitochondria. The presence of this enzyme ensures the functioning of the tricarbonic acid cycle (TCA) at a lack of pyruvate, a basic substrate, and creates the conditions for the pathway of phospho*enol*pyruvate (PEP) produced in glycolysis (Xia and Roberts 1994, Mohanty and Ong 2003, Lemaire *et al.* 2005). After decarboxylation and oxidation by ME, the resulting pyruvate is incorporated into the TCA cycle (Hatch *et al.* 1982, Zhang *et al.* 2001). High metabolic activity may deprive the TCA cycle of the intermediate metabolites. This situation is prevented by anaplerotic reactions which replenish the pool of the TCA cycle intermediates

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Abbreviations: CAM − Crassulacean Acid Metabolism; GOT − glutamate oxaloacetate transaminase (EC 2.14.1.1); NAD(P)-ME, NAD(P) − malic enzymes [EC 1.1.1.38(40)]; PEPC − phospho*enol*pyruvate carboxylase (EC 4.1.1.31); PPDK − pyruvate orthophosphate dikinase (EC 2.7.9.1).

(Gossett *et al.* 1996). All enzymes and transporting metabolites associated with the C_4 cycle occur also in C_3 plants, although they are less active and accumulate in specific tissues (Lai *et al*. 2002). NADP-ME in the C_3 plants contributes to a huge diversity of metabolic pathways in green and non-green tissues of these plants. This enzyme is located in leaves, etiolated tissues, seeds, roots, fruits, bulbs, and in chloroplasts and cytosol. Additionally, NADP-ME increases its activity in the plant response to stresses (Kubien *et al.* 2003).

In the majority of plants the stable content of citrate stays low or high. High irradiance or drought may cause essential changes in the citrate day/night contents. Relatively significant citrate content oscillations have been noted in many CAM plants. The nightly accumulation of citrate or isocitrate is more advantageous in terms of energy than that of malate, which is related to the synthesis and accumulation of ATP during darkness.

In some plants the stress factors such as salinity, drought, or abscisic acid elicit the increase in daily malate content oscillations and shift the metabolism from C_3 to CAM (Chu *et al.* 1990, Cushman and Borland 2002, Haslam *et al.* 2003, Rut *et al.* 2008), but changes in malate and citrate contents or the ME activity in the gametophores of mosses under stress conditions are not documented. The moss species we used for experiments differ in the structure of leaves and environmental requirements. *Hieracium pilosella*, a higher plant species studied, grows in the same environment as *Polytrichum piliferum*. The objective of this study was to check whether, under certain stresses, the contents and accumulation of malate and citrate occur in these plant species. We also tried to link the oscillations in malate content with the changes in ME activity. As the metabolism of malate is alternatively associated with either an uptake or release of CO_2, the amounts of malate were determined in plants which grew in low concentration of CO_2, in order to check whether the changes in malate content are associated with the availability of CO_2. The submergence of plants in water not only restricts the access to oxygen but also alters the nature of the carbon source. In water, the concentration of sources of inorganic carbon (HCO_3^-, CO_3^{2-}) depends on pH, which is essential for photosynthesis. The hypoxia stress was tested by submergence in water for 24 h (12/12 h) at conditions identical to those in the growth chamber. After the prescribed period kept in stress conditions, the contents of malate and citrate, and the activity of ME were determined.

To determine ME content, 0.10–0.15 g of material were mixed with distilled water in a ratio of 1 : 4 and then centrifuged (*MPW-6, MPW-Med,* Warsaw, Poland) for 5 min under 5 000×*g* and kept at 4 °C. The malate content in plants was determined at 19:00 and 07:00 using the method of Möllering (1985). The malate and citrate contents were determined using a spectrophotometer (*Aquarius CE 9500, CECIL,* Cambridge, England) at 340 nm at room temperature. The activity of ME was determined according to Ashton *et al.* (1990). The activity of NAD(P)-ME was measured at 19:00 and 07:00. To determine 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 homogenate was then transferred to Ependorf tubes and centrifuged for 5 min at 5 000×*g*. Then 0.2 cm^3 of a buffer (pH 8.3) 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 at 25 °C for 1 h. The amount of released NADPH was assayed spectrophotometrically at 340 nm. To determine NAD-ME, plant leaves were homogenised with the addition of 2 cm^3 of buffer (pH 7.5) containing 50 mM HEPES, 5 mM DTT, 0.25 % PVP-40 (polyvinylpyrrolidone), 0.5 % Triton, and 2 mM $MnCl_2$. Homogenate was then transferred to Ependorf 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 2 mM 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. The activity of NAD-ME was determined in the same way as for NADP-ME.

Day/night differences in malate contents [mmol kg^{-1}(FM)] in leaves of *P. commune* and *P. piliferum* were small (Fig. 1*A*). This suggests no accumulation of malate in the dark. In leaves of *M. undulatum* and *H. pilosella* malate was accumulated during the night, and the difference between day and night malate contents was ~11.5 mmol kg^{-1}(Fig. 1*A*).

When gametophores of *M. undulatum* were desiccated (loss of ~50 % water) and then watered, the difference in day/night malate contents increased slightly compared with the control to 13 mmol kg^{-1}. However, in the gametophores of *P. piliferum,* this difference was much greater (Fig. 1*A*). At hypoxia, the differences in diurnal oscillations of malate in the gametophores of *M. undulatum* amounted to ~12.5 mmol kg^{-1}, whereas in the leaves of *P. commune* and *H. pilosella* the difference between the periods of light and darkness was only slight (Fig. 1*A*). The greatest difference in day/night malate contents was found in the leaves of *P. piliferum* kept at high irradiance (400 μmol m^{-2} s^{-1}) and was ~12 mmol kg^{-1}. In the leaves of *H. pilosella*, the greatest diurnal oscilations in malate content were found (~28 mmol kg^{-1}; Fig. 1*A*). When the gametophores of the mosses studied were kept in CO_2-free air, the difference in malate content was much lower, ~6 mmol kg^{-1} in *M. undulatum* and ~3 mmol kg^{-1} in *P. piliferum* (Fig. 1*A*). The decline in accumulation of malate may be connected with the decreased activity of PEPC.

The day/night differences in the citrate contents in the leaves of *M. undulatum, P. piliferum*, and *H. pilosella* were very small. The greatest difference was found in the gametophores in *P. commune* (Fig. 1*B*). Diurnal changes

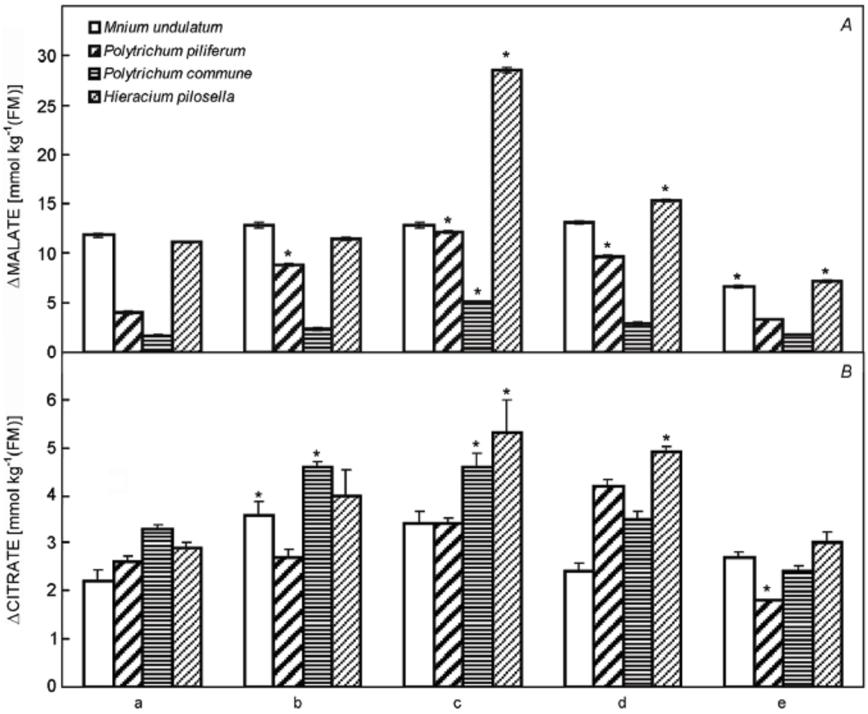

Fig. 1. Diurnal changes in (*A*) malate and (*B*) citrate contents in gametophores kept under control (a), hypoxia (b), high irradiance (c), drought stress (d), and CO_2-free air (e). [*]above bars indicates significance of differences from control plants at $p<0.05$. Means ± SD, $n = 5$.

Table 1. Changes in NADPH and NADH contents [mmol kg^{-1}(FM)] in the leaves of plant species towards the end of the light (L) or darkness (D) periods. *M.u.* – *Mnium undulatum*; *P.p.* – *Polytrichum piliferum*; *P.c.* – *Polytrichum commune*; *H.p.* – *Hieracium pilosella*. Means±SD, *n* = 5.

	Plant	C			Irrad.			Hypox.			Drought			0 CO_2		
		L	D	Δ	L	D	Δ	L	D	Δ	L	D	Δ	L	D	Δ
NADPH	*M.u.*	2.15	2.43	0.28	2.09	2.43	0.34	2.06	2.53	0.47	2.03	2.47	0.44	1.85	1.96	0.11
		±0.05	±0.05	±0.08	±0.08	±0.16	±0.16	±0.09	±0.09	±0.04	±0.07	±0.07	±0.05	±0.06	±0.08	±0.08
	P.p.	1.43	1.59	0.16	1.26	2.08	0.82	1.14	1.76	0.62	1.38	1.94	0.56	1.12	1.20	0.08
		±0.15	±0.13	±0.10	±0.07	±0.09	±0.09	±0.16	±0.06	±0.21	±0.09	±0.12	±0.15	±0.06	±0.08	±0.01
	P.c.	1.40	1.56	0.16	1.50	1.92	0.42	1.73	1.93	0.20	1.77	1.86	0.09	1.39	1.60	0.21
		±0.16	±0.06	±0.11	±0.25	±0.08	±0.33	±0.09	±0.02	±0.11	±0.19	±0.14	±0.05	±0.09	±0.09	±0.06
	H.p.	2.02	2.34	0.32	2.09	3.18	1.09	2.15	2.85	0.70	2.10	2.53	0.43	1.61	1.81	0.20
		±0.17	±0.12	±0.15	±0.11	±0.18	±0.14	±0.04	±0.14	±9.14	±0.09	±0.09	±0.06	±0.8	±0.12	±0.06
NADH	*M.u.*	0.90	1.06	0.16	0.89	1.16	0.27	0.82	0.94	0.12	0.93	1.10	0.17	0.82	0.89	0.07
		±0.15	±0.12	±0.07	±0.12	±0.19	±0.08	±0.04	±0.03	±0.02	±0.05	±0.02	±0.03	±0.13	±0.10	±0.03
	P.p.	1.02	1.16	0.14	0.93	1.14	0.21	0.97	1.16	0.19	1.04	1.22	0.18	0.95	1.03	0.08
		±0.11	±0.10	±0.02	±0.15	±0.08	±0.07	±0.08	±0.01	±0.07	±0.07	±0.06	±0.02	±0.11	±0.12	±0.01
	P.c.	1.81	1.93	0.12	1.89	2.07	0.18	1.82	1.95	0.13	1.55	1.95	0.40	1.82	1.96	0.14
		±0.11	±0.08	±0.04	±0.19	±0.16	±0.04	±0.06	±0.06	±0.01	±0.08	±0.05	±0.12	±0.19	±0.24	±0.05
	H.p.	1.64	1.77	0.13	1.69	1.85	0.16	1.80	1.91	0.11	1.61	1.84	0.23	1.26	1.35	0.09
		±0.10	±0.07	±0.04	±0.29	±0.25	±0.07	±0.15	±0.21	±0.04	±0.08	±0.09	±0.01	±0.14	±0.11	±0.03

in citrate contents in plants kept under stress were markedly lower than those in malate contents. The diurnal oscillations during the drought stress were evident in the leaves of *P. piliferum* and *H. pilosella* (4.2 and 4.9 mmol kg^{-1}, respectively), whereas in the gametophores of *M. undulatum* and *P. commune* the changes were small (Fig. 1*B*). Hypoxia increased citrate contents in the gametophores of *P. commune* and *M. undulatum*, whereas the oscillations of this compound in *P. piliferum* were similar to those in the control plants (Fig. 1*B*). The changes in citrate content under irradiance stress were highest in the leaves of the *H. pilosella* and *P. commune*, whereas in the leaves of *M. undulatum* and *P. piliferum* the fluctuations in citrate content did not deviate significantly from those in control plants (Fig. 1*B*).

The activity of NADP-ME was higher in the leaves of *M. undulatum* and *H. pilosella* than in *P. commune* and *P. piliferum* as demonstrated by the changes in NADPH after the respective periods of irradiation and darkness, whereas the activity of the NAD-ME isoform was low (Table 1). Under stresses, the activity of NADP-ME isoform underwent much greater changes than that of NAD-ME. The activity of NADP-ME under drought was large in leaves of *P. piliferum,* whereas the activity of this enzyme in *M. undulatum* was slight (Table 1). The somewhat higher activity of this enzyme under drought stress was found in the gametophores of *P. commune* (Table 1).

The amount of NADPH produced under hypoxia in leaves as a result of ME activity increased in *P. piliferum* and *H. pilosella.* In the gametophores of *M. undulatum,* this amount was similar to the values in the control (Table 1).

The activity of NADP-ME under irradiance stress was highest in the leaves of *H. pilosella* and *P. piliferum*, whereas in *M. undulatum* leaves the increase in the activity of NADP-ME was slight, while NAD-ME activity increased slightly in the *M. undulatum* gametophores (Table 1).

Keeping plants in CO_2-free air suppressed the activities of both NADP-ME and NAD-ME. The greatest changes in the activities of these enzymes occurred in the leaves of *M. undulatum* and *P. piliferum* (Table 1).

The increased accumulation of malate in the studied moss species and the increased activity of enzymes involved in the metabolism of malate, particularly NADP-ME, correlated with the plant response to adverse environment. Increased malate content may indicate an elevated PEPC activity in the leaves of gametophores in mosses kept under of elevated CO_2 concentration (unpublished data). However, the activity of this enzyme in the gametophores was lower than in the leaves of typical C_4, such as maize (Hatch *et al.* 1982). A full C_4 cycle or CAM would require the increased activity of PPDK, whose activity has not yet been noted in mosses. Malate may also serve as an additional source of carbon under stress, whilst it provides a further option of CO_2 accumulation (Heber *et al.* 2001, Crecelius *et al.* 2003). The increased amount of malate in the studied moss species might be linked to the photosynthetic carboxylation of CO_2. The drop in malate content in CO_2-deprived air suggests that this is a source of carbon, after CO_2 is freed by ME; as this process occurs in the same way in C_4 and CAM plants. The slight accumulation of citrate during the night is advantageous in terms of energy management, being related to synthesis and accumulation of ATP. The changes in NADP-ME activity in *H pilosella* at drought stress could be associated with the role of this enzyme in closing stomata through catabolism of the malate.

The damage to green plants during hypoxia is associated with the decreased availability of not only oxygen, but also CO_2 dissolved in water. The lowered concentration of oxygen in aquatic environment is associated with a slow diffusion of this gas in water and with its low solubility (Edwards *et al*. 2001, Mohanty and Ong 2003, Rzepka *et al.* 2003). The activity of PEPC was also higher than that of ME in plants whose roots were exposed to hypoxia. This was connected with intense synthesis of malate by PEPC more than with the rate of metabolism of this compound by ME. Additionally, ME was very active during several minutes of hypoxia (Edwards *et al*. 1998, Lemaire *et al.* 2005). The increase in malate content in submerged moss gametophores could therefore be related to activities of key enzymes of with the metabolism of malate, namely pyruvate dehydrogenase and PEPC. In aquatic plants grown in low CO_2 concentrations, the malate contents increase at the expense of intermediates of the Calvin cycle. Moreover, the compensating CO_2 concentration drops, whereas the activities of PEPC and NADP-ME increase. Both the changes in NADP-ME activity and the increase in malate content in the studied gametophores of mosses and higher plant could result from changes in metabolism similar to those in aquatic plants. The lack of changes in NAD-ME activity in plants affected by stresses might show that this enzyme does not participate in their response to stresses. The rate of photosynthesis in submerged gametophores shows a decrease after a short period of submersion, while respiration is not significantly affected (Rzepka *et al.* 2005).

In the nature, both *P. piliferum* and *H. pilosella* grow under a high irradiance. It can not be excluded that the oxidation stress may also occur in the studied species. The night accumulation of malate could be one of the adaptations to the adverse conditions during irradiance stress. The activity of NADP-ME in higher plants increases in response to damage at high irradiance. Pyruvate, another product of the NADP-ME activity, can be used in the synthesis of ATP in the respiration process or be used as a substrate for the synthesis of PEP that may, in turn, be used as a substrate to produce compounds essential in defensive reactions. In higher aquatic plants, the induction of NADP-ME and PEPC was prompted under high irradiance.

An intensified synthesis of malate was found when *P. piliferum* and *H. pilosella* plants were kept under increased irradiance and thus it was possible to avoid an accumulation of ROS. This development depends on the quantities of accumulated and transported malate, which may constitute a reserve of reduced carbon equivalents. In the leaves of *P. piliferum, M. undulatum*, and *H. pilosella*, the highest diurnal oscillations of malate and citrate were found during the response to high irradiance, desiccation, or submergence in water. We think that increased amounts of malate and citrate, and increased NADP-ME activity under stresses are important in their adaptation strategy and enable a major ‘plasticity’ of the changes in the metabolism of these species.

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