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ORIGINAL PAPER

Pathogen-induced changes in malate content and NADP-dependent malic enzyme activity in C_3 or CAM performing *Mesembryanthemum crystallinum* L. plants

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Abstract Changes in malate concentration and activity of NADP-dependent malic enzyme were observed as the effect of *Botrytis cinerea* infection of C_3 or CAM-performing *Mesembryanthemum crystallinum* plants. Biotic stress applied on C_3 plants led to increase in malate concentration during the night and in consequence it led to increase in Δ-malate (day/night fluctuations) in infected leaves on the 2nd day post infection (dpi). It corresponded with induction of additional isoform of NADP-malic enzyme (NADP-ME3). On the contrary, CAM-performing *M. crystallinum* plants exhibited decrease in malate concentration and decay in its diurnal fluctuations as a reaction to *B. cinerea* infection. This correlated with significant decrease in activities of NADP-malic enzyme isoforms on the 2nd dpi as well as no fluctuations in their activities on the 9th dpi. Presented results point out to differences between C_3 and CAM plants in the direction of changes in primary metabolism providing energy, reducing equivalents and carbon skeletons for defense responses to halt the pathogen growth.

Keywords *Botrytis cinerea* · CAM · Ice plant · Malate · NADP malic enzyme

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Introduction

Mesembryanthemum crystallinum L. (ice plant) is an annual halophytic plant that in its natural habitat shows seasonal shift from C_3 type of photosynthesis to Crassulacean Acid Metabolism (CAM) (Winter and Holtum 2007) During CAM cycle, malic acid is accumulated in the vacuole as a central intermediary in the process of carbon assimilation during the dark and then it is metabolized in the following light period (Cheffings et al. 1997). One of the important malate metabolizing enzyme is NADP-malic enzyme (NADP-ME, L-malate: NADP oxidoreductase [oxaloacetate decarboxylating], EC 1.1.1.40) which produces after malate decarboxylation apart from pyruvate also CO_2 to be used in carbon fixation by ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco). NADP-ME is known as one of CAM-related enzymes and in *M. crystallinum* at least one CAM-specific isogene for NADP-ME was reported (Cushman 1992).

Due to ability to change the mode of CO_2 fixation under specific environmental conditions like, for example, salinity, *M. crystallinum* was extensively studied in context of engagement of different abiotic stressors in photosynthetic metabolism transition (Bohnert and Cushman 2000). One of the earliest cellular responses to different abiotic stimuli is production of reactive oxygen species (ROS) via consumption of oxygen in a phenomenon called oxidative burst (Gill and Tuteja 2010). Because oxidative burst is also the first symptom of stress resulting from interaction with pathogens (Baker and Orlandi 1995) we assumed that an ice plant might become an interesting model for studies of changes in plant metabolic pathway in response to biotic stress. It is commonly known that plants use ROS as secondary messengers in signal transduction cascades in processes as diverse as mitosis, tropisms, cell death and plant development (Foyer and Noctor 2005). Therefore, it

might be supposed that ROS might also play function in C_3-CAM transition since CAM type is a component of the defense strategy for ameliorating oxidative burst evoked by stress factor (Miszalski et al. 1998; Hurst et al. 2004).

Botrytis cinerea is the most comprehensively studied plant pathogens (Williamson et al. 2007). As a necrotroph it induces ROS accumulation in plant tissues leading to oxidative burst that kills plant cells and enables pathogen to spread within the tissue. The production of hydrogen peroxide during plant–*Botrytis* interaction occurs at the site of infection, as well as in the surrounding uninfected cells, demonstrating their role as signaling molecule (Asselbergh et al. 2007).

The induction of the wide array of defense mechanisms against stress factors involves changes in metabolic activities to redistribute energy and metabolites to defense response. In this context primary metabolism that provides building blocks and energy for the biosynthesis of defense compounds was not studied very extensively. On the basis of fluctuations in the malate concentration and changes in activities of malate-transforming enzymes upon stress conditions, it is suggested that malate metabolism plays an important function in plant defense (Martinoia and Rentsch 1994). Malate is present in all cell types and can be accumulated to level as high as 350 mM and it exhibits multiplicity of functions as an essential carbon storage molecule, an intermediate of the tricarboxylic acid (TCA) cycle, pH regulator, compound controlling efficiency of nutrient uptake and involved in stomatal movement (Fernie and Martinoia 2009). Moreover, it is thought to facilitate apoplastic NADH production that stimulates production of hydrogen peroxide needed to sustain lignification process which is one of well-documented reaction of plants to biotic stress (Baker and Orlandi 1995). This defense pathway inhibiting the infection, similarly as strengthening of host cell walls via cross-linking of glycoproteins and hypersensitive response (HR), is stimulated by ROS overproduction (Fernie and Martinoia 2009). NADP-ME apart from producing CO_2 from malate can be also involved in mechanisms producing NADPH for synthesis of ROS that are produced to kill or damage pathogens (Drincovich et al. 2001). Pyruvate, another product of NADP-ME reaction, can be used for obtaining ATP in the mitochondria and may serve as a precursor for synthesis of phosphoenolpyruvate (PEP). PEP is also utilized in the shikimate pathway, leading to the synthesis of aromatic amino acids including phenylalanine, the common substrate for lignin and flavonoid synthesis.

The main goal of experiments described in the present paper was to investigate the differences in defense reaction of *M. crystallinum* plants, performing C_3 or CAM, to *B. cinerea* infection with respect to malate concentration and NADP-malic enzyme activity.

Materials and methods

Plant material and pathogen infection

Mesembryanthemum crystallinum L. plants were grown from seeds in soil culture under a 12-h photoperiod at 25/17°C (day/night), 60/80% RH (relative humidity) and irradiance of 200–250 μmol quanta m^{-2} s^{-1} (PAR—photosynthetically active radiation; $\lambda = 400$–700 nm). Two sets of plants were used for the experiments: plants irrigated with water (C_3) and plants irrigated for 2 weeks with 400 mM NaCl (CAM).

B. cinerea culture was a kindly gift of Prof. M. Skłodowska (University of Łódź, Poland). One leaf of third whorl of *M. crystallinum* plants in C_3 and in CAM stage was inoculated with solution without fungi spores (MOCK) or it was inoculated with solution containing *B. cinerea* spores (INF). The injection solution contained 5 mM glucose, and 2.5 mM KH_2PO_4 for mock inoculated leaves or it contained 2×10^6 spores ml^{-1} in 5 mM glucose and 2.5 mM KH_2PO_4. Two injections of about 0.2 ml each were applied with a syringe into the bottom leaf surfaces on the both sites of the main vein.

Biochemical analysis

Analyses were performed on extracts isolated from leaves harvested at 8:00 a.m. and at 8:00 p.m. from plants after 2- and 9 days post infection (dpi) with inoculation solution containing spores of fungi or with inoculation solution without spores. Samples were described as follows: CON—untreated control leaves, MOCK—mock inoculated leaves without fungal spores, INF—leaves inoculated with *B. cinerea* spores.

Malate content

Concentration of malate was measured as described by Möllering (1974). Day/night differences of malate concentrations were expressed as Δ-malate ($[mM] = [malate]_{dawn} - [malate]_{dusk}$) in the cell sap.

NADP-ME activity

Procedure presented by Maurino et al. (2001) was used for assay of NADP-ME activity. Plant tissues (0.5 g) were homogenized in 1.5 ml of 100 mM Tris–HCl buffer (pH 7.8) containing 1 mM dithiothreitol, 1 mM EDTA and 5 mM $MgCl_2$; then 0.02 g of polyvinylpolypyrrolidone was added and the homogenate was centrifuged at $14{,}000 \times g$ for 15 min at 4°C. The protein concentration in obtained extracts was determined according to Bradford 1976 using the BioRad protein assay (Bio-Rad) with BSA

as a standard. Protein fractions were stored at 80°C until further use. Native gel electrophoresis was performed according to Laemmli 1970. Protein samples (15 μg) were loaded on polyacrylamide gels (10%). Bands corresponding to NADP-ME activity were detected after incubation of gels in a solution of 100 mM Tris–HCl (pH 7.4) containing 10 mM L-malate, 10 mM $MgCl_2$, 2 mM $NADP^+$, 0.1 mg/ml nitroblue tetrazolium and 5 μg/ml phenazine methosulfate at room temperature.

Densitometric analysis

Gels images were analyzed using BIOPRINT ver.99 computer software (Vilber-Lourmat, France). The activities of all isoforms were evaluated in arbitrary units (AU) corresponding to the area under densitometric curve.

Statistical analysis

All the experiments were performed in triplicates and statistical analyses were done using the STATISTICA 7.1 program. To determine individual treatment effects at the $P \leq 0.05$ level of probability we used a multifactorial ANOVA followed by Tukey's test.

Results

Results of malate concentration measurements indicated that inoculation with *B. cinerea* spores had caused changes in this metabolite content in C_3 and in CAM plants (Fig. 1). Significant increase in malate content at 8:00 a.m. was observed 2 and 9 dpi with solution of *B. cinerea* spores in C_3 performing *M. crystallinum* leaves (Fig. 1a). Stronger accumulation of malate during the night period than during the day led to increase in Δ-malate in C_3 plants inoculated with *B. cinerea* spores (Fig. 1b). In CAM-performing plants significant decrease in malate content was observed 2 dpi, and this significant drop in malate concentration was very strong on the 9th dpi in all investigated leaves (MOCK and INF) in comparison with untreated leaves (CON) (Fig. 1c). Together with decrease in malate concentration decrease in fluctuations in malate content was noted and they were the most evident on the 9th dpi in comparison with untreated leaves (CON) (Fig. 1d).

Activities of three NADP-ME isoforms were visualized on native polyacrylamide gels (Figs. 2, 3). They were described on the basis of their mobility on polyacrylamide gels from the fast to the slowest as NADP-ME3, NADP-ME2 and NADP-ME1. Activities of particular isoforms were expressed depending on metabolic stage as well as plant treatment with *B. cinerea* spores. In samples collected from C_3-performing plants activity of NADP-ME3 was found to be the strongest in leaves collected after 2 dpi with solution containing *B. cinerea* spores (INF) as well as with solution without spores of fungi (MOCK). Moreover, inoculation of C_3-performing *M. crystallinum* plants with *B. cinerea* spores led to the induction of NADP-ME1 on the 2nd dpi additionally to unchanged NADP-ME3 activity in comparison with untreated (CON) and mock-inoculated leaves (MOCK) (Fig. 2a). Leaves of C_3 plants collected on 9 dpi exhibited decrease in activity of NADP-ME3 that was the most evident after inoculation with *B. cinerea* spores. Moreover, in addition to NADP-ME3 isoform the presence of very low active NADP-ME2 was shown in leaves collected 9 dpi at 8:00 p.m. (Fig. 2a, b).

In CAM-untreated leaves isoforms of NADP-ME exhibited diurnal fluctuations in their activities being the highest at 8:00 a.m. and the lowest at 8:00 p.m. Diurnal fluctuation of total NADP-ME activity was also shown in leaves collected on 2 dpi with solution of spores (INF) or solution without *B. cinerea* spores (MOCK); however, decrease in activities of isoforms was found. Activities of both NADP-ME3 and NADP-ME1 isoforms returned to the activity level found in control material (CON) on the 9th dpi with *B. cinerea* spores; however, no fluctuations in their activities were found (Fig. 3a, b).

Discussion

In *M. crystallinum*, a CAM-inducible plant, an age-dependent or salinity-triggered CAM might be observed and with the onset of CAM day/night differences in the concentration of malic acid (Δ-malate) are observed (Libik et al. 2004). Therefore, high Δ-malate can be considered as the most prominent parameter indicating CAM induction. However, in some C_3 plants malate concentrations might be also changed under a variety of physical, chemical and biotic stresses (Martinoia and Rentsch 1994; Lance and Rustin 1984; Schwachtje and Baldwin 2008). In *M. crystallinum* plant, changes in diurnal malate fluctuations contribute to resistance to abiotic stress factors, including salinity, drought, high light intensity, low temperature and anoxia (Taybi et al. 2002). In our experiments it was studied whether *M. crystallinum* might also react similarly to biotic stress factor. In previous studies it was found that *M. crystallinum* in C_3 as well as in CAM stage exhibited resistance to *B. cinerea*. Development of fungi in both metabolic leaf types was limited to 48 h after inoculation and localized in the site of primary necrosis described as symptoms of hypersensitive-like response (HR) (Kuźniak et al. 2010; Libik-Konieczny et al. 2011). On the basis of diurnal fluctuations in malate concentration measured in our experiments, it was concluded that biotic stressor as *B. cinerea* led to the increase in diurnal oscillations of

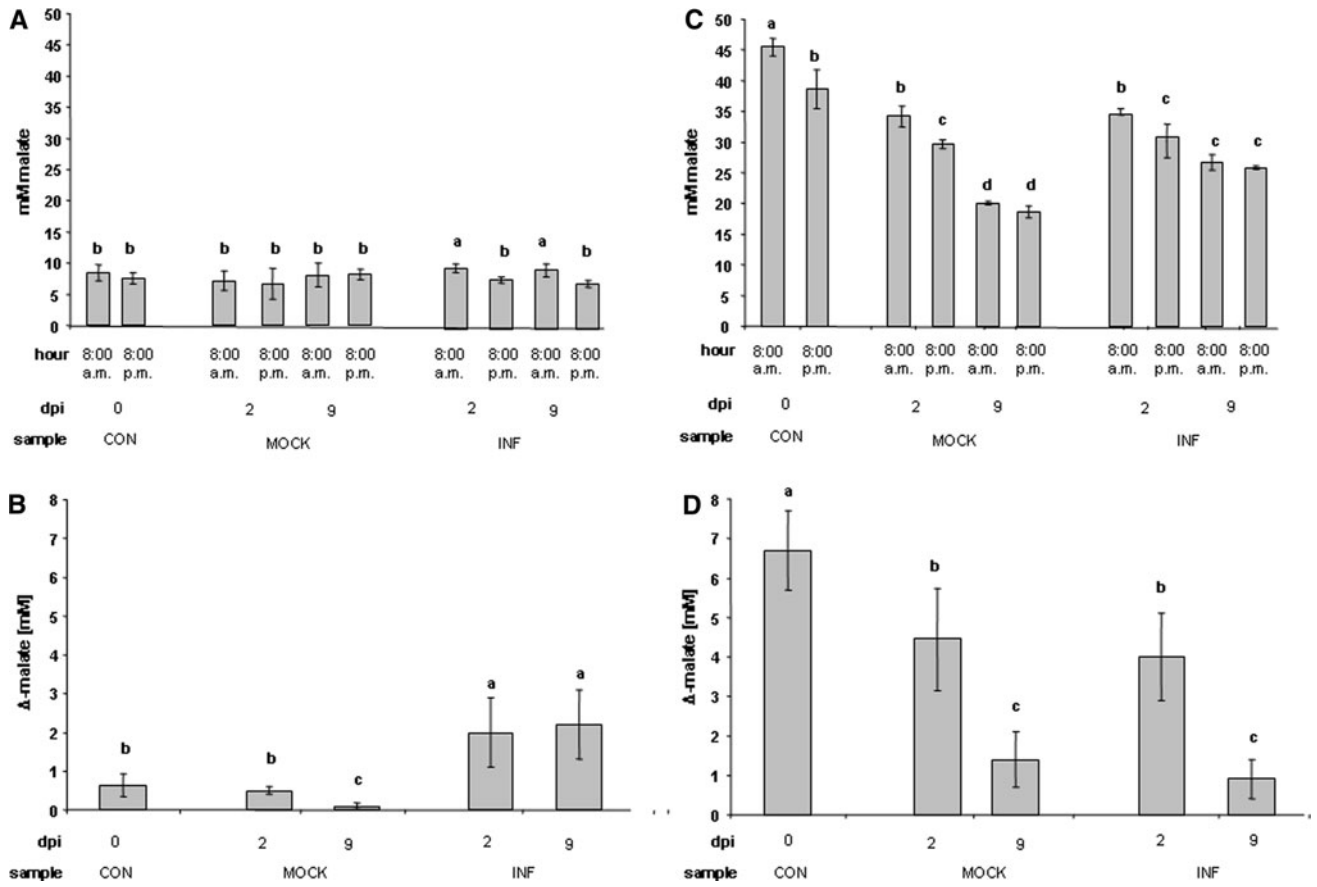

Fig. 1 Malate concentration and Δ-malate in *M. crystallinum* leaves in C_3 stage (**a**, **b**) or CAM stage (**c**, **d**) in control samples (CON), in leaves injected with inoculation solution without *B. cinerea* spores (MOCK) and in leaves infected with *B. cinerea* (INF)

malate content in the range 2 mM. in C_3-performing *M. crystallinum* plants just 2 dpi with *B. cinerea* spores (Fig. 1a, b). On the contrary, in CAM plants decrease in malate content and in diurnal malate fluctuations (Δ-malate) was described; however, similar changes in malate content were caused by inoculation with spores of fungi as well as by inoculation with solution without spores (Fig. 1c, d). Differences in the direction of changes in malate fluctuations between C_3 and CAM plants might result from differences in their abilities to accumulate ROS leading to oxidative stress. It is known that malic acid acts as an inductor of apoplastic peroxidase to produce ROS taking part in defense against pathogen attack. Therefore, this source of ROS might be reduced in CAM plants since, as it was stated before, they have to deal with higher salt concentration inducing oxidative stress in comparison with C_3 plants (Baker and Orlandi 1995).

Malate decarboxylation by NADP-malic enzyme is associated with plant responses to biotic or abiotic stresses (Maurino et al. 2001; Chi et al. 2004; Smeets et al. 2005; Liu et al. 2007). This metabolic pathway was speculated to be involved as energy source for plant defense through the generation of pyruvate and NADPH (Casati et al. 1999; Schaaf et al. 1995). The NADP-ME is widely distributed among all types of plants. This enzyme is encoded by a multigene family and different isogenes of NADP-ME were described in CAM, C_4 as well as in C_3-performing plants (Gerrard Wheeler et al. 2005). Differences in the expression of particular NADP-ME isoform occur not only among plant species, but also in various tissues and in different developmental stages (Chi et al. 2004). Non-photosynthetic NADP-ME isoform was studied with details in maize roots and etiolated leaves (Maurino et al. 2001). This isoform is under transcriptional or post-transcriptional regulation by different signals related to plant defense response such as fungal elicitors and most likely it is engaged in mechanisms of defense against different stressors. Recently, it was also reported that the activity of NADP-ME of *Nicotiana tabacum* L. in infected leaves increased by 5–6 times (Ryšlava et al. 2003). Our results showed that NADP-ME in *M. crystallinum* plant is present at least in three isoforms differing in their expression in investigated samples. Similarly, three isoforms of NADP-ME were characterized in maize: 62 kDa form, which is implicated in C_4 metabolism

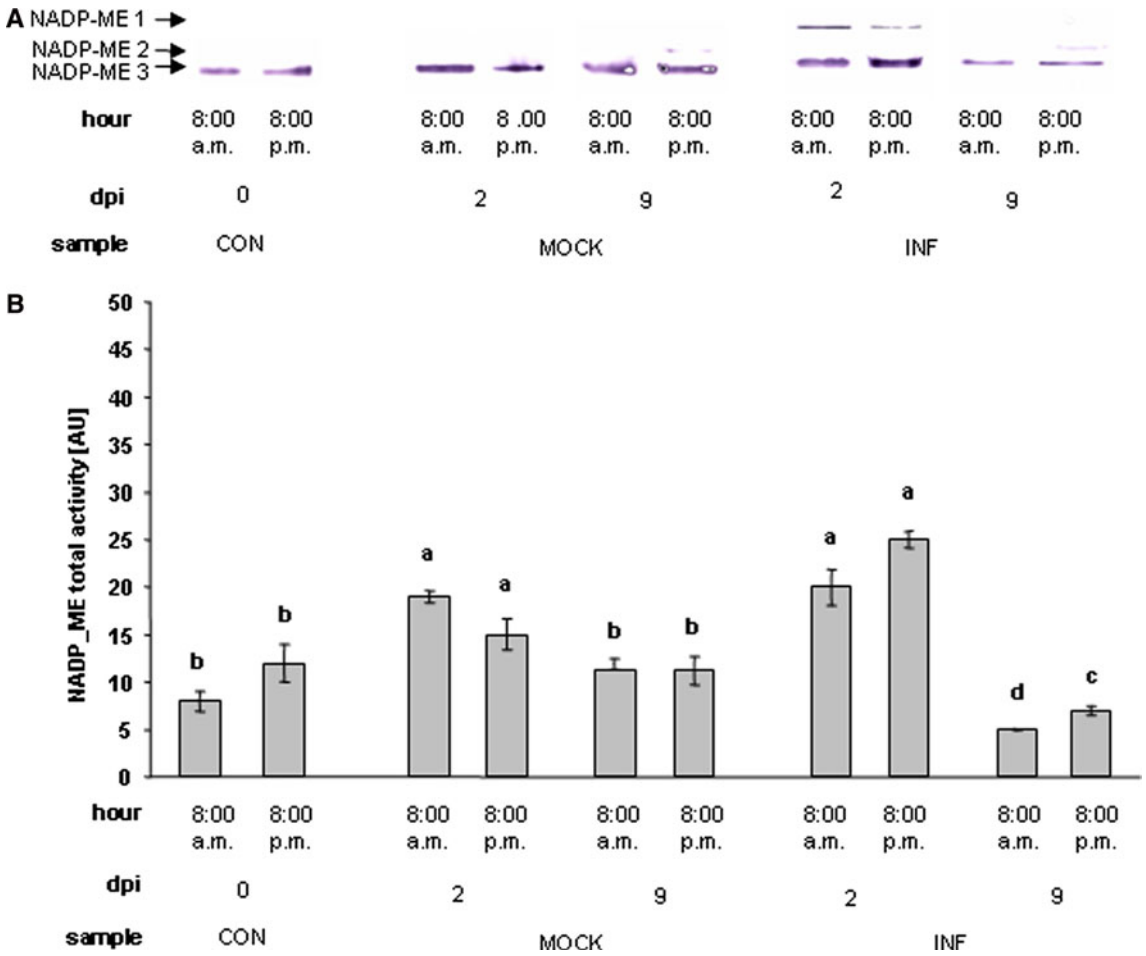

Fig. 2 Visualisation of NADP-ME activity in protein extracts of *M. crystallinum* leaves in C_3 stage after *B. cinerea* infection, separated on native PAGE (**a**); each lane was loaded with 15 μg of protein. Plots exhibiting results of densitometric analysis of visualized bands (**b**). Total NADP-ME activity was calculated on the basis of the densitometric analysis of activity stained gels and expressed in arbitrary units (AU) corresponding to area under densitometric curve. All values were compared statistically at $P \leq 0.05$ and different letters above the bars present significant differences between treatments

and predominant in green leaves, 66 kDa non-photosynthetic isoform assembles as a dimmer and 72 kDa non-photosynthetic form of the protein, present mainly in etiolated leaves (Maurino et al. 2001; Saigo et al. 2004). The existence of isoforms of NADP-ME, which differ in their expression during induction of CAM, was already found in *M. crystallinum* plant. These isoforms were distinguished by their electrophoretic mobility and antigenic differences. NADP-malic enzyme extracted from C_3-performing leaves migrated more rapidly than that extracted from CAM-mode leaves (Saitou et al. 1994). Moreover, evidences for the presence of non-photosynthetic isoform of NADP-ME were also described. This form, isolated from roots, migrated on non-denaturating gels as rapidly as the one extracted from leaves of CAM plants (Drincovich et al. 2001). In our experiments C_3 as well as CAM-performing *M. crystallinum* plants all exhibited activity of isoform described as NADP-ME3. This one is supposed to play an important role in photosynthetic metabolism generating CO_2 and reducing power in chloroplasts, where Calvin cycle operates. Infection with *B. cinerea* spores led to the induction of NADP-ME1 in C_3-performing plants. The same isoform was described in CAM-performing *M. crystallinum* plants and it exhibited fluctuations in its activity being present at the beginning of light period and disappearing at the beginning of dark period. This could indicate its important function in malate decarboxylation to produce CO_2 during the day. This pattern of NADP-ME isoform's activity in CAM-performing *M. crystallinum* was disrupted due to application of stress factor. Both fungal and mock inoculations led to decrease in activity of all NADP-ME isoforms at 2 dpi followed by increase in their activity on the 9th dpi to the level comparable with this found in control plants; however, no fluctuations were observed suggesting their engagement rather in defense mechanisms than in photosynthetic metabolism.

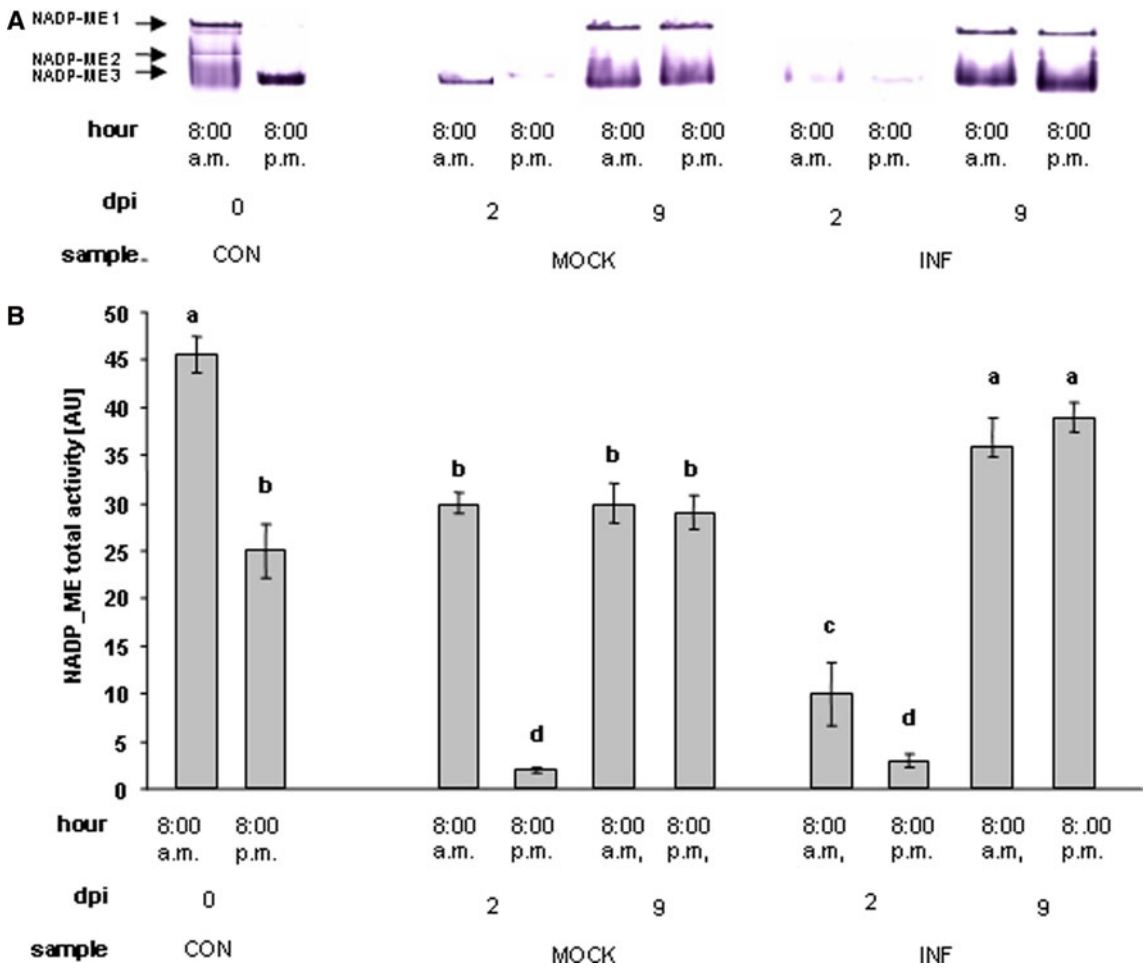

Fig. 3 Visualization of NADP-ME activity in protein extracts of *M. crystallinum* leaves in CAM stage after *B. cinerea* infection, separated on native PAGE (**a**); each lane was loaded with 15 μg of protein. Plots exhibiting results of densitometric analysis of visualized bands (**b**). Total NADP-ME activity was calculated on the basis of the densitometric analysis of activity stained gels and expressed in arbitrary units corresponding with area under densitometric curve. All values were compared statistically at $P \leq 0.05$ and different letters above the bars present significant differences between individual treatments

Taken together, it could be concluded that differences in amount of malate stored in plant performing C_3 or CAM might indicate the changes in primary metabolism that provides building material and energy for biosynthesis of defense compounds, suggesting an alternative role of malate metabolism in plant defense through the action of NADP-ME.

Author contribution Libik-Konieczny M. designed research, wrote a paper and has a primary responsibility for final content, Surówka E., Nosek M. and Goraj S. conducted research and analyzed data, Miszalski Z. participated in writing a paper. All authors have read and approved the final manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

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