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Nitrogen metabolism-related enzymes in *Mesembryanthemum crystallinum* after *Botrytis cinerea* infection

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Abstract

We compared C_3 and CAM (crassulacean acid metabolism) states in *Mesembryanthemum crystallinum*, a facultative CAM species, with respect to the involvement of phospho*enol*pyruvate carboxylase (PEPC) and nitrogen metabolism-related enzymes in plant response to *Botrytis cinerea* infection. The enzyme activities were monitored both in pathogen-inoculated 2nd leaf pair and non-inoculated 3rd leaf pair. The control activities of most studied enzymes were dependent on the mode of photosynthesis. Compared to C_3 plants, those performing CAM exhibited higher PEPC, nitrate reductase (NR), and deaminating glutamate dehydrogenase (NAD-GDH) activities but lower glutamine synthetase (GS) and alanine aminotransferase (ALT) activities. Regardless of the mode of photosynthetic carbon assimilation, the plants responded to infection with enhancement of PEPC and inhibition of NR activities in the inoculated leaves. Whereas the activity of GS remained unaffected, those of all glutamate-yielding enzymes, namely ferredoxin-dependent glutamate synthase (Fd-GOGAT), aspartate aminotransferase (AST), ALT, and aminating glutamate dehydrogenase (NADH-GDH) were altered after infection. However, the time-course and extent of the observed changes differed in C_3 and CAM plants. In general, CAM plants responded to infection with an earlier increase in PEPC and Fd-GOGAT activities as well as later inhibition of NR activity. Contrary to C_3 plants, in those performing CAM the activities of PEPC, Fd-GOGAT, NADH-GDH, and AST in the non-inoculated 3rd leaf pair were similarly influenced by infection as in leaves directly inoculated with the pathogen. This implies that the local infection induced an alteration of carbon/nitrogen status in healthy upper leaves. This reprogramming resulting from changes in PEPC and nitrogen metabolism-related enzymes was C_3- and CAM-specific.

Additional key words: crassulacean acid metabolism, glutamate dehydrogenase, grey mould, ice plant, nitrate reductase, phospho*enol*pyruvate carboxylase.

Introduction

Upon infection, the plant and the pathogen develop a complex relationship affecting almost all metabolic processes in the host cells, including carbon and nitrogen metabolism. Although the role of primary metabolism in plant defense against pathogens has evoked considerable interest, this problem still awaits clarification. As concern to nitrogen metabolism, two contrasting effects were observed during pathogenesis: *1*) the nitrogen status of plant tissues influenced pathogen infection and colonization and *2*) the plant nitrogen metabolism was manipulated by the pathogen for its own benefit (Snoeijers *et al*. 2000, Bolton and Thomma 2008). Depending on the lifestyle of the invading pathogen, changes in nitrogen metabolism during pathogenesis can either promote plant defense or support pathogen growth and infection development (Liu *et al*. 2010, Seifi *et al*. 2013).

The main metabolic pathway by which inorganic N is converted into organic N in plants consists of sequential enzymatic reactions catalyzed by nitrate reductase

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Abbreviations: ALT - alanine aminotransferase; AST - aspartate aminotransferase; CAM - crassulacean acid metabolism; dai - day after inoculation; EDTA - ethylenediaminetetraacetic acid; Fd-GOGAT - ferredoxin-dependent glutamate synthase; GS - glutamine synthetase; NAD-GDH - NAD-dependent glutamate dehydrogenase; NADH-GDH - NADH-dependent glutamate dehydrogenase; NADH-GOGAT - NADH-dependent glutamate synthase; NiR - nitrite reductase; NR - nitrate reductase; PEPC - phospho*enol*-pyruvate carboxylase; TCA cycle - tricarboxylic acid cycle.
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(NR, EC 1.6.6.1), nitrite reductase (NiR, EC 1.7.7.1), glutamine synthetase (GS, EC 6.3.1.2), and glutamate synthase (GOGAT; EC 1.4.1.14 for NADH-GOGAT and EC 1.4.7.1 for ferredoxin-dependent glutamate synthase, Fd-GOGAT). In this process, NR-mediated nitrate reduction to nitrite is considered to be the rate limiting step (Masclaux-Daubresse *et al.* 2010).

Under environmental stresses, an additional pathway mediated by glutamate dehydrogenase (GDH, EC 1.4.1.2) is induced to control the flux of nitrogen (Cebeci *et al*. 2008, Miyashita and Good 2008). This enzyme catalyzes the reversible reductive amination of 2-oxoglutarate to yield glutamate and acts to avoid ammonia accumulation under stress (Skopelitis *et al*. 2006). However, *in vivo* GDH also deaminates glutamate and fuels the tricarboxylic acid cycle (TCA cycle) with 2-oxoglutarate. Alanine aminotransferase (ALT, EC 2.6.1.2) and aspartate aminotransferase (AST, EC 2.6.1.1) catalyzing the reversible transfer of amino group from glutamate to pyruvate/oxaloacetate to form 2-oxoglutarate and alanine/aspartate, respectively have also been shown to be involved in mechanisms that allow plants to survive under stress conditions (Gajewska and Skłodowska 2009, Gao *et al*. 2013). The aminotransferase-catalyzed amination of 2-oxoglutarate provides plant cells with glutamate which is of central importance for nitrogen remobilization during pathogenesis (Seifi *et al*. 2013).

In plants, the interdependence of primary carbon and nitrogen metabolism has been extensively studied and the pivotal role of photosynthesis in nitrogen assimilation is well known (Foyer and Noctor 2002, Nunes-Nesi *et al*. 2010, Gawronska and Niewiadomska 2015). The flexible coordination of nitrogen assimilation with carbon metabolism is considered important for a broad spectrum of physiological and developmental processes in plants (Sun *et al*. 2013) as well as for plant response to stressful environmental conditions, including biotic stress.

The metabolic rearrangements in plant cells during plant-pathogen interactions contribute to plant defense and they are usually manifested by downregulation of the primary metabolism, mainly photosynthesis and other processes supporting plant growth (Scharte *et al*. 2005, Bilgin *et al*. 2010). The energy and metabolite resources saved by downregulation of the primary metabolism can be diverted to defense processes. Our previous studies demonstrated that the infection-induced changes in photochemical activity could be also dependent on the type of photosynthetic carbon assimilation. In *Mesembryanthemum crystallinum*, a facultative C_3-CAM (crassulacean acid metabolism) plant, we observed different patterns of *Botrytis cinerea*-induced changes in the photochemical activity in C_3 and CAM plants. While in CAM plants, the photochemical activity visualized by chlorophyll fluorescence imaging was decreased, in C_3 plants, it remained unchanged (Gabara *et al*. 2012). These infection-induced changes could alter the flow of carbon skeletons and reduced equivalents to interacting pathways of nitrate nitrogen assimilation.

Phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31), indispensable for primary CO_2 fixation in C_4 and CAM plants, serves a variety of non-photosynthetic functions. In all photosynthetic plant types, PEPC is involved in the coordination of carbon and nitrogen assimilation by replenishing the TCA cycle with oxaloacetate which is converted into 2-oxoglutarate used in nitrogen assimilation (Doubnerová and Ryšlavá 2011).

The aim of this study was to analyze the effects of *B. cinerea* infection on nitrogen metabolism enzymes in leaves of *M. crystallinum* with respect to the type of photosynthetic carbon assimilation. The activities of nitrogen metabolism-related enzymes, namely NR, GS, Fd-GOGAT, GDH, ALT, and AST, as well as PEPC involved in coordination of carbon and nitrogen assimilation were monitored in plants operating in the C_3 and CAM mode. This enzymatic response was studied both locally (in the inoculated leaves) and systemically (in the non-inoculated upper leaves). To our knowledge, this is the first report on the influence of infection on nitrogen metabolism-related enzymes in *M. crystallinum*, the model C_3-CAM plant.

Materials and methods

Plant material and pathogen infection: The ice-plant (*Mesembryanthemum crystallinum* L.) was grown from seeds in a growth chamber as described earlier (Kuźniak *et al*. 2013). After the appearance of the 3rd leaf pair, one set of plants was irrigated with 0.4 M NaCl to induce CAM (CAM plants) while another was further irrigated with water (C_3 plants). The induction of CAM in NaCl-treated plants was detected by measuring diurnal malate fluctuations assumed as a hallmark of CAM (Kuźniak *et al*. 2013). A 12-d treatment with 0.4 M NaCl induced CAM with Δ malate in the range of 10 - 15 mM. Thereafter, leaves of the 2nd leaf pairs of C_3 and CAM plants were inoculated by infiltration with *Botrytis cinerea* spore suspension (1×10^6 spores cm^{-3}) supplemented with 5 mM glucose and 2.5 mM KH_2PO_4 according to Kuźniak *et al*. (2013). Control plants were infiltrated with 5 mM glucose and 2.5 mM KH_2PO_4. Leaves of control and infected C_3 and CAM plants were taken for analyses 1, 2, and 3 d after inoculation (dai). We analyzed samples from the 2nd leaf pairs (inoculated leaves) as well as from the 3rd leaf pairs (non-inoculated upper leaves) prepared as described by Kuźniak *et al*. (2013). Samples were collected in the middle of the light period.

Microscopy analysis of fungal infection: Samples of infected leaf tissues (0.5 × 0.5 cm) were taken from the infiltrated areas 20 h after inoculation, squashed in distilled water and examined under a light microscope (*Leica*, Wetzlar, Germany) equipped with digital camera.

Alternatively, the samples were stained with 0.01 % (m/v) trypan blue in lactophenol. The plant material was transferred into a test tube, covered with staining solution, placed in a heated water bath and boiled for one minute. The staining solution was removed by boiling in lactoglycerol (glycerol + 90 % lactic acid + distilled water in volume proportions 1:2:5). The samples were mounted in glycerol, observed under the light microscope, and photographed.

Preparation of leaf extracts: The major leaf veins were removed from the leaves and the samples for biochemical analyses (0.5 g) were prepared from the remaining leaf lamina (Kuźniak *et al.* 2016). For estimation of NR and Fd-GOGAT activities, fresh tissue was homogenized (1:5, m/v) in the ice cold mortar using 50 mM potassium phosphate buffer, pH 7.5, containing 2 mM EDTA, 10 mM KCl, 14 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 3.58 M ethylene glycol. For estimation of PEPC, GS, GDH, ALT, and AST activities, fresh tissue was homogenized (1:5, m/v) in the ice cold mortar using 50 mM Tris-HCl buffer pH 7.6 containing 1 mM EDTA, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM dithiothreitol, and 0.5 % (m/v) polyvinylpyrrolidone (PVP). The homogenates were centrifuged at 20 000 *g* for 20 min.

Enzyme assays: The activity of PEPC was measured in a coupled enzymatic assay with malate dehydrogenase and was expressed in μmol NADH oxidized per mg protein per minute as described by Gajewska *et al.* (2013). Nitrogen metabolism-related enzymes, *i.e.* NR, GS, Fd-GOGAT, GDH, ALT, and AST were determined according to the previously published protocols (Gajewska and Skłodowska 2009). NR activity was assayed by measuring the content of NO_2^- and was expressed in nmol NO_2^- formed per mg protein per minute. The activation state of NR (reflecting how much of the enzyme is in the non-phosphorylated active form) was given as a percentage ratio between actual (measured in the presence of Mg^{2+}) and maximum (measured in the presence of EDTA) NR activity. Fd-GOGAT activity was assayed with methyl viologen as electron donor and was expressed in nmol glutamate formed per mg protein per minute. GS activity was estimated using the transferase assay based on reaction of glutamine with hydroxylamine and was expressed in μmol γ-glutamylhydroxamate formed per mg protein per minute. NADH-GDH and NAD-GDH activities were assayed spectrophotometrically by monitoring the oxidation of NADH or reduction of NAD and were expressed in nmol NADH oxidized or NAD reduced per mg protein per minute, respectively. ALT activity was assayed in the alanine→pyruvate direction by coupling the reaction with NADH oxidation by lactate dehydrogenase. AST activity was assayed in the aspartate→oxaloacetate direction by coupling the reaction with NADH oxidation by malate dehydrogenase. ALT and AST activities were expressed in μmol NADH oxidized per mg protein per minute.

Protein content was determined according to Bradford (1976) using standard curves prepared for bovine serum albumin.

Data analysis: The data are means from 6 independent experiments ($n = 6$). In each experiment, two plants were used to prepare one leaf sample, and a single sample was analyzed for each treatment. Sample variability was given as a standard deviation (± SD) of the mean. The significance of differences between control and infected plants as well as between C_3 and CAM plants was determined by Student's *t*-test using the *Statistica*® software. Differences at $P < 0.05$ were considered significant.

Results

In the inoculated leaves of C_3 plants, *B. cinerea* conidia germination and the hyphal growth were facilitated when compared to CAM plants. The light micrographs taken 20 h after inoculation showed that within the inoculum-infiltrated areas in C_3 leaves, the germinating spores formed long, proliferating hyphae filled with dense cytoplasm whereas in CAM plants, the germ tubes were short and swollen (Fig. 1).

The PEPC activity in control CAM *M. crystallinum* plants was several-fold higher than in C_3 plants (Fig. 2). The difference was more pronounced in the 2nd leaf pair showing on the 1st day of experiment about 9-fold higher PEPC activity in CAM plants compared to C_3 plants. At the same time in the 3rd leaf pair of CAM plants, the activity of this enzyme was about 7-fold higher than in C_3 plants. In both C_3 and CAM plants, infection with *B. cinerea* resulted in an increase in PEPC activity in the 2nd leaf pair (3rd dai). Compared to the control, it was increased by 37 and 27 % in C_3 and CAM plants, respectively. In the non-inoculated 3rd leaf pair as early as 1 dai both C_3 and CAM plants showed induction of PEPC activity by 40 and 37 %, respectively. On the 3rd dai the activity of this enzyme was increased only in CAM plants, by 58 % over the control level.

In the 3rd leaf pair, the control NR activity was significantly higher in CAM plants as compared to C_3 plants (Fig. 3). In both C_3 and CAM plants, infection led to a decline in NR activity in the 2nd leaf pair. In C_3 plants decreases in NR activity were observed on the 1st and 3rd dai by 36 and 42 %, respectively. In CAM plants a detrimental effect of infection on NR activity was visible starting from the 2nd dai, when it was decreased by 26 % compared to the control. On the 3rd dai, the activity of this enzyme was reduced by 45 %. In the non-inoculated 3rd leaf pair, no significant changes in NR activity were observed in response to infection except for

the 2nd dai when in C_3 plants it was increased by 46 % compared to the control.

Infection did not significantly influence NR activation state in *M. crystallinum* leaves except for the 2nd leaf pair of C_3 plants which on the 3rd dai showed its increase by 27 % compared to the control (Fig. 3). The GS activities in control 2nd and 3rd leaf pairs of CAM plants were up to 20 % lower than those in C_3 plants (Fig. 4). Neither in C_3 nor in CAM plants infection changed GS activity in leaves. Infection with *B. cinerea* differently influenced Fd-GOGAT activity in the 2nd leaf pair of C_3 and CAM plants (Fig. 4). Whereas C_3 plants showed a 26 % decrease in this enzyme activity on the 1st dai and subsequently a 25 % increase on the 3rd dai, in CAM plants a 40 % induction found on the 1st dai was followed by a 31 % inhibition on the 2nd dai. In the non-inoculated 3rd leaf pair, an infection-induced 50 % increase in Fd-GOGAT activity was observed in CAM plants on the 1st dai.

The C_3 plants did not show significant changes in NADH-GDH activity in response to infection (Fig. 5). On the contrary, in CAM plants, infection resulted in a decrease in NADH-GDH activity on the 1st dai, both in the 2nd and 3rd leaf pairs, by 31 and 44 % compared to the

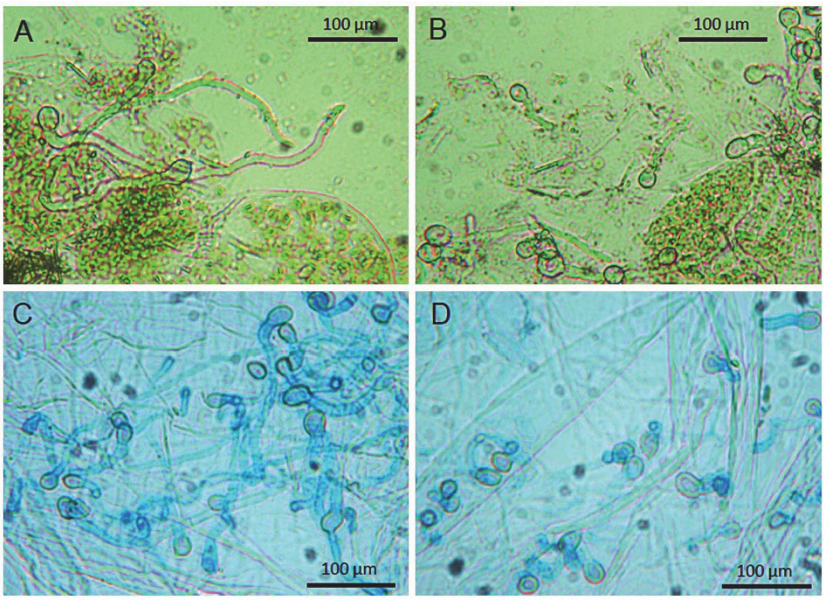

Fig. 1. *Botrytis cinerea* conidia germination and hyphae growth in infected *Mesembryanthemum crystallinum* leaves (the 2nd pair). Light micrographs were taken 20 h after inoculation. *A* and *C* - C_3 plants; *B* and *D* - CAM plants; Trypan blue staining (*C*,*D*). Images are representative of three independent experiments using a minimum of three C_3 and CAM plants.

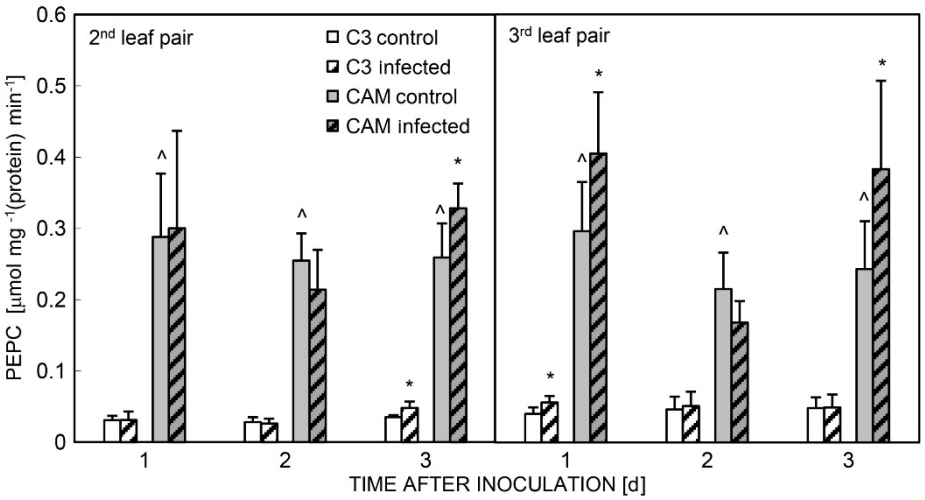

Fig. 2. Effect of *Botrytis cinerea* infection on phospho*enol*pyruvate carboxylase (PEPC) activity in the inoculated 2nd and non-inoculated 3rd leaf pairs of C_3 and CAM *Mesembryanthemum crystallinum* plants. Means ± SDs, $n = 6$, * indicate a significant difference between control and infected plants at $P < 0.05$, ^ indicate a significant difference between C_3 and CAM plants at $P < 0.05$.

control, respectively. On the 3rd dai an increase in NADH-GDH was found only in the 2nd leaf pair, by 37 % compared to the control. The NAD-GDH activity (deaminating) was about 2 to 4-fold higher than NADH-GDH activity (aminating), depending on the leaf age and type of metabolism (Fig. 5). Irrespective of the leaf age, the control NAD-GDH activity in CAM plants was over 2-fold higher compared to C_3 plants. Infection did not significantly alter NAD-GDH activity in *M. crystallinum* leaves.

The control C_3 plants exhibited significantly higher ALT activity compared to CAM plants, both in the 2nd and 3rd leaf pairs (Fig. 6). Infection of C_3 plants resulted in an increase in ALT activity in the 2nd leaf pair on the 1st and the 2nd dai, by 33 and 39 % over the control, respectively. The 2nd leaf pair of CAM plants showed a 44 % increase in ALT activity on the 3rd dai. The ALT activity in the 3rd leaf pair in both C_3 and CAM plants was not influenced by *B. cinerea* infection.

Compared to ALT, AST exhibited higher activity in control plants (Fig. 6). The 2nd leaf pair of C_3 and CAM plants responded differently to infection, the former showing a 25 % decrease in AST activity on the 1st dai and the latter a 44 % increase on the 3rd dai. The activity of AST in the non-inoculated 3rd leaf pair of C_3 plants increased on the 2nd dai while in those of CAM plants on the 3rd dai, by 34 % and 43 % compared to the control, respectively.

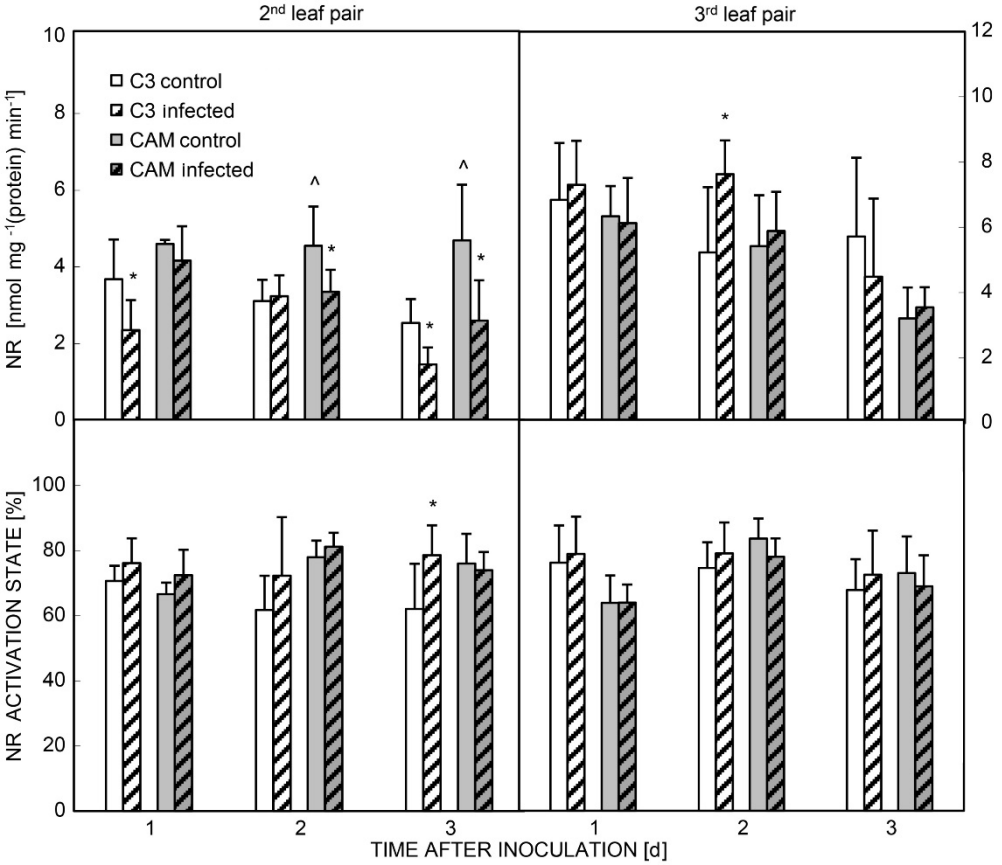

Fig. 3. Effect of *Botrytis cinerea* infection on nitrate reductase (NR) activity and NR activation state in the inoculated 2nd and non-inoculated 3rd leaf pairs of C_3 and CAM *Mesembryanthemum crystallinum* plants. Means ± SDs, $n = 6$, * indicate a significant difference between control and infected plants at $P < 0.05$, ^ indicate a significant difference between C_3 and CAM plants at $P < 0.05$.

Discussion

Our earlier studies showed that both C3 and CAM *M. crystallinum* plants are resistant to *B. cinerea*. Within 48 h after inoculation, symptom development is facilitated in C3 plants, however, the further *B. cinerea* growth is restricted within necrotic lesions resulting from a hypersensitive-like resistance response in plants representing both C3 and CAM metabolism types (Kuźniak *et al*. 2010, Libik-Konieczny *et al.* 2011). We found that NaCl in the concentration used for CAM induction influences the *B. cinerea* hyphae morphology (Kuźniak *et al*., 2011). However, the restriction of symptom development is probably not due to NaCl action as it occurs in both C3 and CAM plants. Moreover, NaCl in the concentration range from 0.1 to 0.8 M, does not

inhibit *B. cinerea* growth *in vitro* (Kuźniak *et al*. 2010).

As photosynthetic carbon metabolism is intimately linked with nitrogen metabolism (Nunes-Nesi *et al*. 2010), and modulation of enzymes of carbon and nitrogen metabolism could be an important regulatory point of carbon/nitrogen interaction, the present study addressed changes in activities of PEPC and nitrogen metabolism enzymes in C_3 and CAM *M. crystallinum* plants after *B. cinerea* infection. In our study, PEPC activity in the leaves of CAM plants was several-fold higher than in C_3 plants. Compared to C_3 plants, those performing CAM showed significantly higher activities of NR and NAD-GDH but lower activities of GS and ALT which could reflect CAM-specific changes in the C/N status of plant cells. Increased activity of NR observed in some halophytic species grown under saline conditions has been attributed to high nitrogen requirements arising from the accumulation of nitrogenous osmoprotectants, such as proline or glycine betaine, in their tissues (Stewart and Rhodes 1978). Despite difference in the control level of NR activity, C_3 and CAM *M. crystallinum* plants showed a similar activation state of this enzyme.

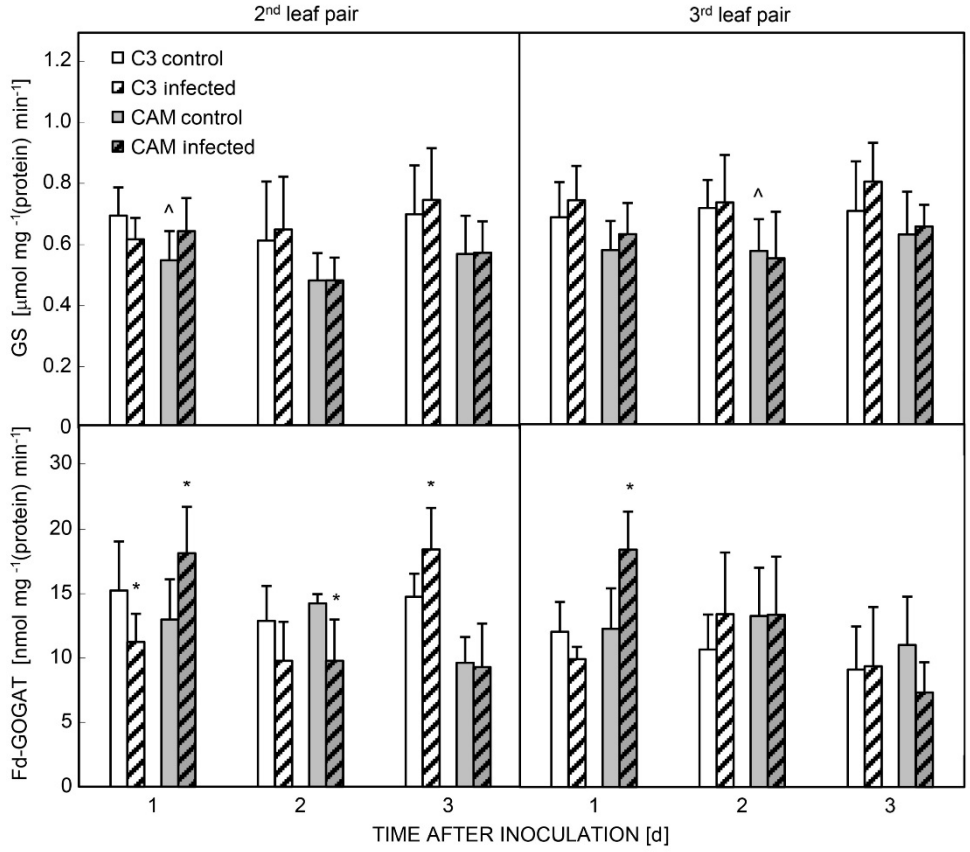

Fig. 4. Effect of *Botrytis cinerea* infection on glutamine synthetase (GS) and ferredoxin-dependent glutamate synthase (Fd-GOGAT) activities in the inoculated 2nd and non-inoculated 3rd leaf pairs of C_3 and CAM *Mesembryanthemum crystallinum* plants. Means ± SDs, $n = 6$, * indicate a significant difference between control and infected plants at $P < 0.05$, ^ indicate a significant difference between C_3 and CAM plants at $P < 0.05$.

In C_3 and especially in CAM plants, the activity of NAD-GDH markedly exceeded that of NADH-GDH indicating that glutamate dehydrogenase served mainly catabolic function, as it is reported for majority of higher plants (Lea and Miflin 2003). Higher NAD-GDH activity in control CAM plants than in C_3 plants reflected intensive glutamate deamination resulting in the release of 2-oxoglutarate. This reaction can be used to funnel the C skeleton of glutamate into the TCA cycle.

Despite different PEPC activity in control C_3 and CAM *M. crystallinum* plants, they responded similarly to *B. cinerea* infection showing an increase in this enzyme activity on the 3rd dai. These results indicate that in plants challenged by the pathogen, regardless of the type of photosynthetic carbon metabolism, β-carboxylation-related processes could be involved in reprogramming plant metabolism from growth and development to defense (Doubnerová and Ryšlavá 2011). Besides providing CO_2 for Calvin cycle, the infection-inducible PEPC promotes the anaplerotic replenishment of C_4-dicarboxylic acids utilized for energy and biosynthetic metabolism which could be advantageous for plants under biotic stress. Comparing to our findings a much higher increase in PEPC activity was reported for plants subjected to viral infection (Ryšlavá *et al*. 2003).

Regardless of the mode of photosynthesis, the

infected leaves showed a significant decrease in NR activity, however, the pattern of changes depended on the type of metabolism performed. Inhibitory effect of infection on NR activity was observed earlier in C_3 plants, which might contribute to the faster leaf colonization by *B. cinerea*. The observed decrease in NR activity seems to be unrelated to the post-translational modifications of the enzyme molecule by phosphorylation, which was evidenced by unchanged or even increased NR activation state found in *B. cinerea*-infected leaves. Besides decline in enzyme synthesis or its increased degradation (Botrel and Kaiser 1997), a decrease in maximum NR activity found in our study might have resulted from the shortage of a reductant in infected *M. crystallinum* leaves. Nitrate assimilation is sensitive to the availability of NADH, which comes, among others, from the photosynthetic electron transport chain (Foyer and Noctor 2002). We found that in C_3 plants, the photochemical activity remained unchanged after *B. cinerea* infection whereas in infected leaves of CAM plants significant decreases in maximal photosystem II quantum yield (characterized by variable to maximum chlorophyll fluorescence ratio, F_v/F_m), photochemical quenching (qP), and non-photochemical quenching (NPQ) were observed 3 dai (Gabara *et al*. 2012). We suggest that the depressed photochemical activity may be important for limiting nitrogen assimilation *via* NR in infected CAM leaves found in this study. The infection-induced NR activity decrease in both C_3 and CAM plants could be, at least 3 dai when PEPC activity increased, the effect of feedback regulation of NR by malate. As shown by Müller *et al*. (2001), malate represses NR at the transcription and enzyme activity levels but does not affect enzyme activation *via* reversible phosphorylation. However, NR activity increase in the non-inoculated 3rd leaf pair of infected C_3 plants 2 dai could reflect the stimulatory effect of photorespiration on nitrate reduction (Nunes-Nesi *et al*. 2010).

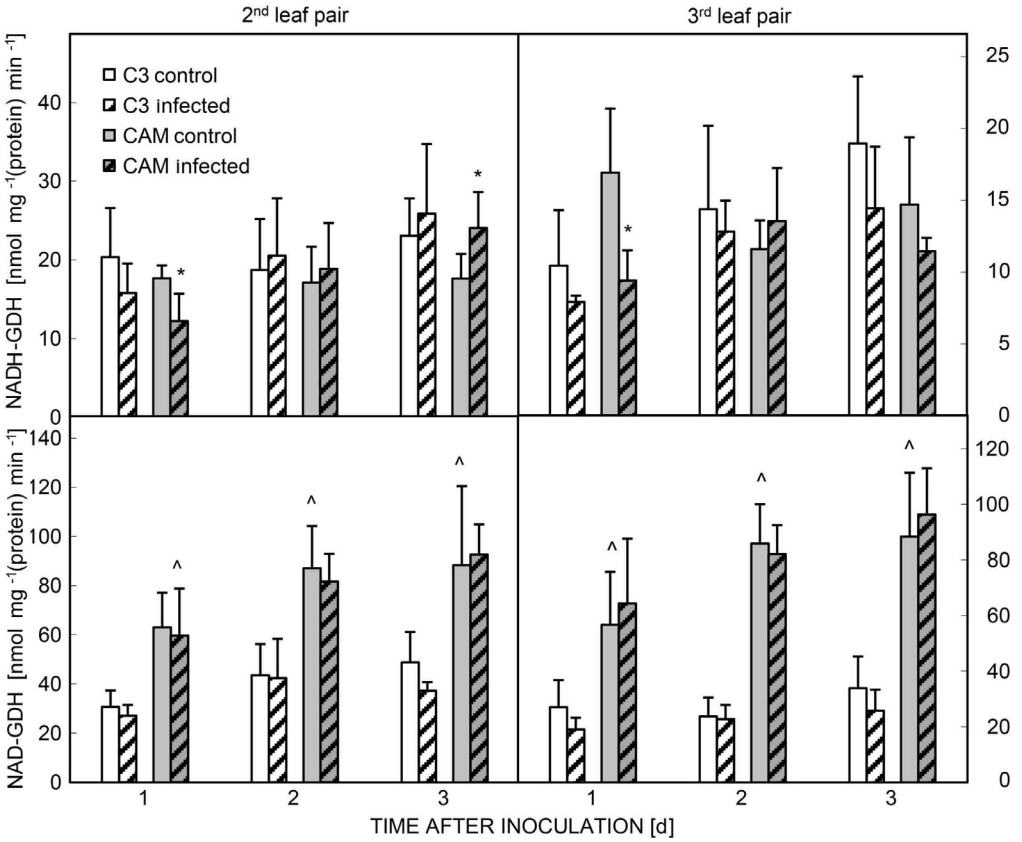

Fig. 5. Effect of *Botrytis cinerea* infection on NADH-dependent glutamate dehydrogenase (NADH-GDH) and NAD-dependent glutamate dehydrogenase (NAD-GDH) activities in the inoculated 2nd and non-inoculated 3rd leaf pairs of C_3 and CAM *Mesembryanthemum crystallinum* plants. Means ± SDs, $n = 6$, * indicate a significant difference between control and infected plants at $P < 0.05$, ^ indicate a significant difference between C_3 and CAM plants at $P < 0.05$.

Amino acids and amino acid-derived metabolites play multiple roles in plant resistance to pathogens (Liu *et al*. 2010, Zeier 2013). The depletion of amino acids, especially glutamine, enhances defense reactions in *Arabidopsis* (Liu *et al*. 2010). As in our study, the GS activity in the inoculated 2nd leaves remained unchanged, one can speculate that this might have helped to limit *B. cinerea* infection. In accordance with the postulated key role of glutamate in plant-pathogen interactions (Seifi *et al*. 2013), we found infection-

induced changes in activities of all glutamate-yielding enzymes, namely ALT, AST, NADH-GDH, and Fd-GOGAT. In both C_3 and CAM plants, increased Fd-GOGAT activity was concomitant with *B. cinerea* restriction in infected leaves. The increased Fd-GOGAT activity in leaves of infected CAM plants found 1 dai, when the spore germinating rate slowed down, could indicate the importance of glutamate recycling shortly after inoculation for the defense strategy against *B. cinerea*. In line with this assumption, in inoculated C_3 leaves, *B. cinerea* invasion was restricted later (2 - 3 dai) (Kuźniak *et al*. 2010) and Fd-GOGAT induction was observed only 3 dai. The potent requirements for glutamate recycling in infected leaves could also be met by aminotransferases operating in the catabolic direction. The involvement of ALT and AST depended on the type of photosynthetic carbon metabolism, however, in both C_3 and CAM plants their induction compensated for the decrease in Fd-GOGAT activity in the course of infection. In the inoculated leaves of C_3 plants, ALT was significantly increased 1 and 2 dai whereas in CAM plants, ALT and AST were induced only 3 dai. The enhanced ALT activity supported alanine catabolism in cells of infected C_3 leaves undergoing *B. cinerea*-induced programmed cell death (Kuźniak *et al*. 2013). Given the absence of programmed cell death events in CAM plants (Kuźniak *et al*. 2013), the increased catabolism of alanine and aspartate found in these plants 3 dai may provide pyruvate and oxaloacetate for the TCA cycle to funnel the energy-demanding metabolic pathways. Besides their potential anaplerotic functions, aminotransferases can also be crucial for activation of resistance against pathogens, as shown for specific *Arabidopsis* aminotransferase generating a putative amino acid-derived signal mediating multi-component local and systemic defense responses (Song *et al*. 2004). Moreover, AST provides substrate oxaloacetate for phospho*enol*pyruvate carboxykinase, which besides being involved as decarboxylase in CO_2 concentrating mechanism in CAM plants, plays a role in nitrogen metabolism (Leegood and Walker 2003, Delgado-Alvarado *et al*. 2007)

As adequate metabolic adjustment at the whole organism level is crucial for plant survival under stress, it can be assumed that metabolic information on nitrogen status of the infected leaf is transferred between organs and drives metabolic adjustments, *e.g.*, carbon/nitrogen balance, in non-inoculated parts of the plant (Coruzzi and Zhou 2001, Rojas *et al*. 2014). In leaves, NADH-NR and PEPC along with sucrose phosphate synthetase were

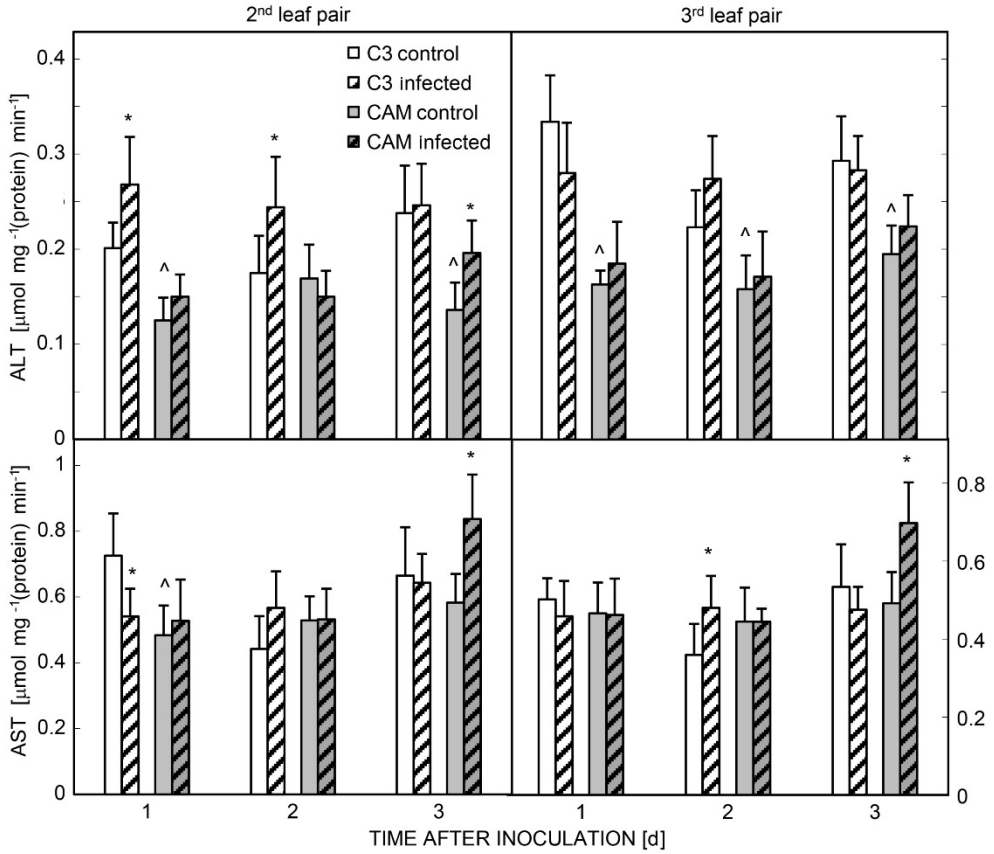

Fig. 6. Effect of *Botrytis cinerea* infection on alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in the inoculated 2nd and non-inoculated 3rd leaf pairs of C_3 and CAM *Mesembryanthemum crystallinum* plants. Means ± SDs, $n = 6$, * indicate a significant difference between control and infected plants at $P < 0.05$, ^ indicate a significant difference between C_3 and CAM plants at $P < 0.05$.

postulated to be important in carbon and nitrogen interaction (Champigny 1995). We found that in infected CAM plants, changes in activities of Fd-GOGAT and NADH-GDH on the 1st dai as well as in PEPC and AST on the 3rd dai induced in the inoculated 2nd leaf pair were mirrored in the non-inoculated 3rd leaf pair. However, in C_3 plants, the responses of inoculated and non-inoculated leaves were not parallel as far as PEPC, AST, and NR activity changes were analyzed. Enzyme activity changes found in the non-inoculated 3rd leaf pair of infected C_3 plants were not observed in the inoculated 2nd leaf pair (AST, NR), or they exhibited a different time course (PEPC). Nevertheless, these results suggest that local infection changed the carbon/nitrogen status in healthy upper leaves by mechanisms involving PEPC and enzymes of nitrogen metabolism to a degree which was specific to the type of photosynthetic CO_2 assimilation. Given the location of PEPC, AST (Aubry *et al*. 2011), and Fd-GOGAT (Chang *et al*. 2012) in bundle sheath cells, and the role of vascular tissues in systemic stress responses in plants, including biotic stress (Vlot *et al*. 2008), activity changes of these enzymes found in healthy leaves of infected plants may be important in maintenance the carbon/nitrogen balance throughout the plant. In stressed plants, these changes could contribute to adjustment to a new state of metabolic homeostasis referred as acclimation. It is an attractive possibility that besides being involved in basal carbon and nitrogen metabolism, PEPC and enzymes of nitrogen metabolism may play a role in the establishment of whole plant response to infection, as suggested for PEPC in *Arabidopsis* subjected to abiotic stresses (Sánchez *et al*. 2006). It cannot be excluded, however, that activity changes in nitrogen metabolism-related enzymes found in the non-inoculated leaves of infected plants resulted from infection-triggered changes in allocation of nitrogen resources, which were affected by the type of photosynthetic carbon metabolism (Schultz *et al*. 2013).

In conclusion, our results indicate that the CAM-specific post-inoculation changes manifested by earlier increase in PEPC and Fd-GOGAT activities as well as later inhibition of NR activity could contribute to faster restriction of *B. cinerea* growth found in the infected leaves of those plants. Moreover, in CAM-performing plants, the activities of PEPC, Fd-GOGAT, NADH-GDH, and AST in the non-inoculated 3rd leaf pair were similarly influenced by the pathogen as in the inoculated 2nd leaf pair. This points to CAM-specific, local infection-induced alteration of carbon/nitrogen status in systemic leaves resulting from changes in PEPC and nitrogen metabolism-related enzymes. As this relationship was not observed in C_3 plants where *B. cinerea* growth was somehow facilitated, it can be suggested that the CAM-specific pattern of changes in nitrogen metabolism-related enzymes favours the expression of defense mechanisms.

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