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REGULAR PAPER

The activities of PEP carboxylase and the C_4 acid decarboxylases are little changed by drought stress in three C_4 grasses of different subtypes

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Abstract The C_4 photosynthetic pathway involves the assimilation of CO_2 by phospho*enol*pyruvate carboxylase (PEPC) and the subsequent decarboxylation of C_4 acids. The enzymes of the CO_2 concentrating mechanism could be affected under water deficit and limit C_4 photosynthesis. Three different C_4 grasses were submitted to gradually induced drought stress conditions: *Paspalum dilatatum* (NADP-malic enzyme, NADP-ME), *Cynodon dactylon* (NAD-malic enzyme, NAD-ME) and *Zoysia japonica* (PEP carboxykinase, PEPCK). Moderate leaf dehydration affected the activity and regulation of PEPC in a similar manner in the three grasses but had species-specific effects on the C_4 acid decarboxylases, NADP-ME, NAD-ME and PEPCK, although changes in the C_4 enzyme activities were small. In all three species, the PEPC phosphorylation state, judged by the inhibitory effect of L-malate on PEPC activity, increased with water deficit and could promote increased assimilation of CO_2 by the enzyme under stress conditions. Appreciable activity of PEPCK was observed in all three species suggesting that this enzyme may act as a supplementary decarboxylase to NADP-ME and NAD-ME in addition to its role in other metabolic pathways.

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Abbreviations

BS	Bundle sheath
DTT	1,4-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
LWP	Leaf water potential
M	Mesophyll
MDH	Malate dehydrogenase
NADH	Nicotinamide-adenine dinucleotide (reduced)
NAD-ME	NAD-malic enzyme
NADPH	Nicotinamide-adenine dinucleotide phosphate (reduced)
NADP-ME	NADP-malic enzyme
PEG	Polyethylene glycol
PEP	Phospho*enol*pyruvate
PEPC	PEP carboxylase
PEPCK	PEP carboxykinase
PPFD	Photosynthetic photon flux density
PVP	Polyvinylpyrrolidone
Rubisco	RuBP carboxylase/oxygenase
RuBP	Ribulose-1,5-bisphosphate
RWC	Leaf relative water content
SWC	Soil water content

Introduction

C_4 photosynthesis is characterised by the presence of a CO_2-concentrating mechanism, which involves the initial fixation of CO_2 by phospho*enol*pyruvate carboxylase (PEPC, EC 4.1.1.31) in the mesophyll (M) cells followed

by the decarboxylation of the resulting C_4 acids in the bundle sheath (BS) cells, where the CO_2 released is assimilated by ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39). The increased CO_2 concentration in the BS cells results in low RuBP oxygenation and, consequently, low rates of photorespiration and increased rates of photosynthesis by C_4 plants (Kanai and Edwards 1999). In the present work, the activities of the primary carboxylation and subsequent decarboxylation reactions and the relative contribution of the decarboxylases involved in C_4 photosynthesis were evaluated in three different C_4 grasses exposed to drought stress.

The three biochemical subtypes of C_4 photosynthesis have been defined by the main enzyme responsible for C_4 acid decarboxylation (Gutierrez et al. 1974; Hatch et al. 1975; Hatch 1987): NADP-malic enzyme (NADP-ME, EC 1.1.1.40), NAD-malic enzyme (NAD-ME, EC 1.1.1.39) and PEP carboxykinase (PEPCK, EC 4.1.1.49). Variations to these 'classical' mechanisms of C_4 photosynthesis occur. PEPCK is present in various species of the NADP-ME subtype (Walker et al. 1997; Voznesenskaya et al. 2006) and acts as a supplementary decarboxylase in *Zea mays* (Wingler et al. 1999). Conversely, PEPCK was not found in some other NADP-ME species like *Sorghum bicolor* (Walker et al. 1997; Wyrich et al. 1998). A set of anatomical characteristics is associated with each biochemical subtype (Gutierrez et al. 1974; Prendergast et al. 1987; Dengler et al. 1994), and most C_4 grasses fit clearly into one of these three anatomical–biochemical subtypes, but Hattersley and Watson (1992) distinguished a total of 10 variants of C_4 plants. The three C_4 grasses studied in the present work were classified as belonging to each of the main biochemical subtypes of C_4 photosynthesis: *Paspalum dilatatum* as a NADP-ME species (Usuda et al. 1984), *Cynodon dactylon* as a NAD-ME species (Hatch and Kagawa 1974) and *Zoysia japonica* as a PEPCK species (Gutierrez et al. 1974). Dallisgrass (*P. dilatatum*), bermudagrass (*C. dactylon*) and zoysiagrass (*Z. japonica*) are warm-season species used for turfgrass purposes throughout the world (Brown 1999), and the first two species are also important as forage and cultivated pasture grasses (Jones 1985).

Water scarcity is increasing in many areas of the world, and the understanding of plant responses to drought and the identification of species better adapted to the expected climate changes are crucial to improve water use efficiency. Plant physiological and biochemical responses to water deficit depend on the rate of induction and severity of the stress, the leaf age and state of development, and the species studied (Chaves 1991). Studies with several C_4 grass species of the NADP-ME subtype, including *Zea mays* (Foyer et al. 1998; Saccardy et al. 1996), *Saccharum* sp. (Du, et al. 1996) and *Setaria sphacelata* (Marques da Silva and Arrabaça 2004), showed contradictory responses of the C_4 enzymes involved in the primary carboxylation, PEPC, and in the decarboxylation of malate, NADP-ME. In *Setaria sphacelata* both maximal and physiological activities and the activation state of PEPC increased with slowly induced drought stress (Marques da Silva and Arrabaça 2004). Conversely, in *Saccharum* sp. a linear decrease of the enzyme activity with decreasing leaf water potential (LWP) was observed (Du et al. 1996). In *Zea mays* PEPC maximal activity was either little (Foyer et al. 1998) or not affected (Saccardy et al. 1996) by water deficit. The activity of the decarboxylase NADP-ME decreased in gradually dehydrated leaves of *Setaria sphacelata* (Marques da Silva and Arrabaça 2004) and *Saccharum* sp. (Du et al. 1996) but was not affected by water deficit in *Zea mays* (Saccardy et al. 1996).

The regulation of the C_4 enzymes involved in the carboxylation and decarboxylation reactions was reviewed by Leegood and Walker (1999). In C_4 plants, PEPC is activated in the light by reversible phosphorylation of a serine residue in the N-terminus and regulated allosterically by metabolites (Chollet et al. 1996; Izui et al. 2004). In the C_4 eudicot *Flaveria bidentis*, PEPC phosphorylation is not essential for efficient photosynthesis and the role of this complex type of regulation remains unclear (Furumoto et al. 2007). The phosphorylation is known to increase PEPC activity and makes the enzyme less sensitive to inhibition by L-malate and more sensitive to activation by glucose-6-phosphate (Vidal and Chollet 1997). Studies on drought-induced changes in the PEPC phosphorylation state, judged by the inhibitory effect of L-malate on the enzyme activity, gave contradictory results in *Zea mays*, with either decreased (Foyer et al. 1998) or unchanged (Saccardy et al. 1996) PEPC sensitivity to the inhibitor in dehydrated leaves. In *Sorghum* leaves PEPC phosphorylation increased under salt stress conditions (Garcia-Maurino et al. 2003).

Information on the regulation of the decarboxylating enzymes involved in C_4 photosynthesis under different environmental conditions is scarce. The activity of NADP-ME in the light is modulated by changes in pH and by the concentrations of L-malate and Mg^{2+} (Iglesias and Andreo 1990). The activity of NAD-ME is regulated by adenylates and by the ratio $NADH/NAD^+$, requires Mn^{2+} and is stimulated in the presence of fructose-1,6-bisphosphate and CoA (Murata et al. 1989). PEPCK has an absolute requirement for Mn^{2+} and is regulated by the concentrations of metal ions and ATP (Walker et al. 1997), inhibited by a number of phosphorylated metabolites (Burnell 1986) and in some, but not all, C_4 plants is susceptible to phosphorylation (Walker and Leegood 1996). Adenylates can have an important role in the coordination of NAD-ME and PEPCK activities (Walker et al. 1997). The

decarboxylation step in the C_4 pathway is generally assumed to be non-limiting for photosynthesis; however, the coordination between the primary carboxylation in the mesophyll and the further decarboxylation in the bundle sheath is crucial to maximise the efficiency of the CO_2-concentrating mechanism in C_4 plants. The aims of the present work were to characterise further three grasses of the different C_4 photosynthetic subtypes, to study the relative contribution of each of the three enzymes involved in the decarboxylation of C_4 acids and to assess the response of the carboxylating and decarboxylating enzymes of the C_4 pathway to gradually induced water deficit.

Materials and methods

Plant material and drought stress induction

The C_4 grasses *Paspalum dilatatum* Poir. cv. Raki, *Cynodon dactylon* (L.) Pers var. Shangri-Lá and *Zoysia japonica* Steudel 'Jacklin Sunrise Brand' (produced by Jacklin Seed Company, USA) were grown from seeds and transferred to 1 l pots of peat-free compost (Petersfield Products, Leicester, UK) supplemented with a slow-release fertiliser (Hydro Agri Ltd., Lincs, UK) in a glasshouse. Artificial light was provided whenever the natural light was below a photosynthetic photon flux density (PPFD) of 500 μmol m^{-2} s^{-1} during a 16-h photoperiod. Temperature was maintained at a minimum of 25°C during the day and at 18°C during the night. Each pot contained five plants and was well-watered until the beginning of the drought stress treatment. Water deficit was then imposed on the 'stress' pots by ceasing to provide water, and the 'control' pots were watered once per day. Each pot corresponded to one independent sample, with 12 control and 12 non-watered pots being used per species. The treatments (control versus drought stress) were randomised in a split-plot design with species as the main plots (columns). From the full set, three control (C) and five non-watered (S) samples of each species were selected according to their leaf dehydration level (in order to optimise the range of drought stress intensities) and the corresponding frozen samples used for biochemical measurements.

Leaf samples were collected in the growth environment 4 h after the beginning of the photoperiod, 8–10 days after drought stress induction: *P. dilatatum* was sampled first, *C. dactylon* next and *Z. japonica* last. Five-week-old plants of the two former species and 9-week-old plants of the slow-growing *Z. japonica* were analysed. Plants of each species had an average of ca. 4 (*P. dilatatum*), 5 (*C. dactylon*) or 10 (*Z. japonica*) fully expanded leaves at the beginning of the drought treatment. These numbers increased during the period when water was withheld, such that, at the end of the experiment, when samples were taken for analyses, control and non-watered plants had, respectively, ca. 9 and 8 (*P. dilatatum*), 17 and 15 (*C. dactylon*) or 24 and 22 (*Z. japonica*) fully expanded leaves. Taking into account the different leaf sizes, each sample of *P. dilatatum* consisted of two leaves, while each sample of the other two grasses consisted of five leaves, taken from the same pot. The youngest fully expanded leaf of each plant of *P. dilatatum* and two young fully expanded leaves of each plant of *C. dactylon* or *Z. japonica* were always used. It was assumed that, within each pot, all the young fully expanded leaves were identical in terms of developmental stage, physiological and biochemical properties, and would have experienced the same drought condition. Therefore, leaf sub-samples were taken from each pot: the first was quickly frozen in liquid nitrogen (LN_2) and then stored at −80°C for biochemical assays and the second was used to determine the leaf relative water content (RWC). The fresh (FW), turgid (TW) and dry (DW) weights were measured and used to calculate RWC by the equation $RWC\ (\%) = 100 \times ((FW - DW)/(TW - DW))$ (Catsky 1960). Afterwards, another young fully expanded leaf was taken from each pot for measurement of the LWP using a pressure-chamber (Ritchie and Hinckley 1975). The soil water content (SWC) was determined in three opposite locations in each pot using an HH2 moisture meter with a Theta probe (type ML2x, AT Delta-t Devices Ltd., Cambridge, UK).

Extraction of C_4 enzymes

The frozen leaf samples (0.1–0.4 g FW) were ground in a cold mortar with quartz sand, 1% (w/v) insoluble PVP and 10 (*P. dilatatum* and *C. dactylon*) or 15 volumes (*Z. japonica*) of ice-cold extraction medium containing 50 mM Bicine-KOH pH 8.0, 1 mM EDTA, 5% (w/v) PVP_{25000}, 6% (w/v) PEG_{4000}, 10 mM DTT, 50 mM 2-mercaptoethanol and 1% (v/v) protease inhibitor cocktail (Sigma, St Louis, MO, USA). After grinding to produce a fine suspension, aliquots were taken for total chlorophyll determination, and the remaining homogenate was centrifuged for 3 min at 14,000*g* and 4°C. The supernatant was kept on ice, while sub-samples were taken for measuring each of the enzyme activities at 25°C in continuous assays monitoring absorbance at 340 nm (UV-500, Unicam Ltd., Cambridge, UK, with the software Vision 32). The extraction medium and procedure were optimised in order to achieve the best recovery of the enzymes activities and to ensure that they remained stable for 1 h. Each value presented is the mean of at least two replicate measurements using the same leaf extract. The total chlorophylls content in the leaf homogenates was determined after extraction in 96% ethanol (Wintermans and de Mots 1965).

PEPC activities and sensitivity to effectors

PEPC activity was measured by coupling the carboxylase reaction with malate dehydrogenase (MDH), essentially as described by Bakrim et al. (1992). The reaction mixture (1 ml) for the determination of the maximal activity of the enzyme (V_{max}), under optimal pH and substrate conditions, consisted of 50 mM Hepes-KOH pH 8.0, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.2 mM NADH (Sigma), 10 U MDH (Sigma), 10 mM PEP (Sigma) and 20 µl of crude extract. The physiological activity ($V_{physiol}$) was determined under similar conditions but at pH 7.3 and with 2.5 mM instead of 10 mM PEP. The reaction mixtures, with all the components except NADH, were allowed to equilibrate at 25°C for 1 min before starting the reaction. The activation state of PEPC was calculated as the ratio $V_{physiol}/V_{max} \times 100$.

One sub-sample of the leaf crude extracts was desalted by gel filtration (Sephadex G-25, Pharmacia Biotech, Uppsala, Sweden, in PD-10 columns) in order to assess the effects of the inhibitor L-malate and of the activator glucose-6-phosphate on PEPC physiological activity. The assays were performed in the same conditions as described above for PEPC $V_{physiol}$, using 40 µl of the desalted leaf extract and measuring the activity in the absence and with increasing concentrations of L-malate or glucose-6-phosphate. The inhibition or activation of the enzyme was estimated by the ratio of the activity at each concentration in relation to the activity in the absence of the effector. Inhibition by aspartate was also tested in the leaf extracts of *C. dactylon* and *Z. japonica*. The sensitivity to L-malate was used to assess the PEPC phosphorylation state (Vidal and Chollet 1997).

Activities of C_4 acid decarboxylases

PEPCK was assayed in the carboxylation direction by coupling the reaction with MDH according to Walker et al. (2002) with minor modifications. The reaction mixture (1 ml) for measurement of the enzyme activity under physiological concentrations of divalent ions contained 100 mM Hepes-KOH pH 7.0, 100 mM KCl, 90 mM $KHCO_3$, 4 mM $MgCl_2$, 10 µM $MnCl_2$, 1 mM ADP (Sigma), 0.2 mM NADH, 12 U MDH (Sigma), 5 mM PEP (Sigma), and the reaction was started by the addition of 40 µl of leaf crude extract. Maximal activity of the enzyme from each of the three species was obtained under the same conditions but in the presence of 5 mM instead of 4 mM $MgCl_2$ and 2 mM instead of 10 µM $MnCl_2$ and starting the reaction by the addition of PEP after incubating the enzyme, contained in the leaf crude extracts, at 25°C with all the other components for 3 min. The activation state of PEPCK was calculated as the ratio between the two activities multiplied by 100.

NADP-ME and NAD-ME activities under conditions that approximate the physiological state were determined using the methods described by Ashton et al. (1990) with minor modifications. For NADP-ME activity, the reaction mixture (1 ml) contained 50 mM Hepes-KOH pH 8.0, 10 mM $MgCl_2$, 0.5 mM $NADP^+$ (Sigma), 5 mM L-malate and 40 µl of crude extract. For NAD-ME activity, the reaction mixture (1 ml) contained 50 mM Hepes-KOH pH 7.2, 4 mM $MnCl_2$, 0.1 mM CoA, 4 mM NAD^+ (Sigma), 5 mM L-malate and 40 µl of crude extract. In both cases, the enzymes contained in the leaf crude extracts were incubated at 25°C with all the components except the substrate for 3 min, and the reactions were then started by the addition of L-malate. This procedure was adopted after obtaining greater rates of NADP-ME activity in *P. dilatatum* and NAD-ME activity in *C. dactylon* under these conditions.

Statistical analysis

All the analyses were made using GenStat® 9.2, 2005 (Lawes Agricultural Trust, Rothamsted Research, UK). Regression analysis was applied to model the variation of enzyme activities and activation states with RWC. Non-significantly different ($P > 0.05$) parameters (*t*-tests) in the significant model terms of the regression (*F*-tests, $P < 0.05$) were amalgamated in order to attain parsimony. The resulting best models were plotted, and the parameter estimates with their respective standard errors (SE), the percentage of variance accounted for by the model (R^2), the residuals mean square (s^2) and the degrees of freedom (df) are given with the plots. All the absolute values and percentages presented in the text were calculated in accordance with the regression analysis performed. Residual maximum likelihood (REML) analysis was used to verify if there was a significant effect of each effector on the activity of PEPC. The ratio of the enzyme activity with each concentration of effector in relation to the activity in its absence was calculated for each sample. The significance of the treatments on this ratio was assessed through the Wald test (Welham and Thompson 1997). Subsequently, mean values estimated for the control and drought-stressed plants of each species at the different effector concentrations were compared using *t*-tests on the appropriate degrees of freedom from the REML model and the standard errors of differences (SED) for all possible comparisons. The least significant difference at the 5% level (LSD(5%)) considering all data for each effector is given as a reference.

Results

Drought stress induction

Drought stress was imposed on plants of the three C_4 grass species by ceasing to provide water to previously selected pots, randomly distributed, while continuing to supply water to the control plants. The SWC, in the non-watered pots was four to five times lower than in the control pots of each species (Table 1), resulting in lower values of RWC and LWP in the drought-stressed samples relative to the controls. In previous experiments in which drought stress was imposed by a similar approach to that in the experiment described here, and in which the leaf samples studied were similar, photosynthetic rates were decreased by ca. 15–20% under moderate water deficit (RWC decreased down to 80–85%) in the three species and decreased by ca. 50–60% under severe water deficit in *P. dilatatum* and *Z. japonica* (Carmo-Silva et al. 2008).

PEP carboxylase

PEPC activity was higher in *C. dactylon* than in the other two species (Fig. 1). In all three species, a very slight but significant ($P \leq 0.001$) increase of $V_{physiol}$ was observed with decreasing RWC, whereas V_{max} was not significantly affected by leaf dehydration ($P > 0.05$). PEPC activation state was lower in *P. dilatatum* (<40%) than in the other two species (ca. 80–90%). The activation state of the enzyme increased by ca. 16% in *P. dilatatum* and 6% in *C. dactylon* and *Z. japonica* when RWC decreased to 90%.

PEPC $V_{physiol}$ in the leaves of the three C_4 grasses was significantly affected ($P < 0.001$) by the concentration of L-malate (Fig. 2). The enzyme was less inhibited in the drought-stressed than in the control plants ($P < 0.001$), and the extent of inhibition differed among the three species ($P = 0.047$), being greater in *P. dilatatum* and *Z. japonica* than in *C. dactylon*. The inhibition of PEPC $V_{physiol}$ by aspartate in the two aspartate-forming species (*C. dactylon* and *Z. japonica*) was less pronounced than the inhibition by L-malate, but the enzyme was similarly more inhibited in *Z. japonica* than in *C. dactylon*, and the enzyme from control leaves of both species was more sensitive to the inhibitor than from the dehydrated leaves. The effect of glucose-6-phosphate on PEPC $V_{physiol}$ ($P < 0.001$) was significantly different among the three species ($P < 0.001$), with greatest extent of activation in *P. dilatatum*. The enzyme present in drought-stressed leaves of *C. dactylon* was slightly more activated by glucose-6-phosphate than in the control leaves, whilst for the other two species the activating effect was greater in the fully hydrated leaves. However, the overall difference between control and non-watered samples was not significant ($P > 0.05$).

Table 1 The soil water content (SWC), leaf relative water content (RWC) and leaf water potential (LWP) of well-watered (C) and drought-stressed (S) plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*

Species		*P. dilatatum*	*C. dactylon*	*Z. japonica*
SWC	C	36.7 ± 0.8	36.2 ± 0.9	39.3 ± 0.2
(%, v/v)	S	6.4 ± 0.6	8.0 ± 1.0	7.7 ± 1.2
RWC	C	98.1 ± 0.1	98.4 ± 0.2	98.1 ± 0.5
(%)	S	87.1 ± 2.9	93.1 ± 1.5	93.4 ± 1.0
LWP	C	−0.93 ± 0.02	−0.82 ± 0.04	−0.95 ± 0.04
(MPa)	S	−1.21 ± 0.03	−1.16 ± 0.04	−1.65 ± 0.12

Both control and drought-stressed plants of each species were analysed 8–10 days after ceasing to provide water to the stress pots. The mean values and respective standard errors were calculated from measurements taken with three control and five drought-stressed samples of each species, corresponding to the same pots used for assaying enzyme activities on leaf extracts

C_4 acid decarboxylases

The activity of PEPCK at optimal concentrations of Mg^{2+} and Mn^{2+} was relatively high in all three species (Fig. 3). The activity of the enzyme in the presence of low but physiological concentrations of the divalent ions was much lower in *P. dilatatum*, with an average activation state of 25.7 ± 2.7%, than in *C. dactylon* and *Z. japonica*, with an activation state generally above 80%. PEPCK activity decreased with leaf dehydration only in *C. dactylon* and at physiological concentrations of Mg^{2+} and Mn^{2+}. The activation state of the enzyme was differently affected in *C. dactylon* and *Z. japonica*, decreasing by 8.5% in the former species and increasing by 24% in the latter when RWC decreased to 90%.

In *P. dilatatum* NADP-ME activity decreased by 8% with decreasing RWC down to 90% (Fig. 4). The activity of this enzyme was very low in *C. dactylon* and *Z. japonica* (0.50 ± 0.09 μmol min^{-1} mg^{-1} Chl) and did not change with leaf dehydration in the two species ($P > 0.05$).

The activity of NAD-ME in fully hydrated leaves of *C. dactylon* (~3.8 μmol min^{-1} mg^{-1} Chl) was higher than in *Z. japonica* (1.59 ± 0.11 μmol min^{-1} mg^{-1} Chl) and very low in *P. dilatatum* (0.62 ± 0.11 μmol min^{-1} mg^{-1} Chl) (Fig. 4). NAD-ME activity was only affected by water deficit in *C. dactylon*, decreasing by 8% when RWC decreased to 90%.

Discussion

Effects of leaf dehydration on enzyme activities

Slowly induced leaf dehydration had a similar effect on PEPC from *P. dilatatum*, *C. dactylon* and *Z. japonica*

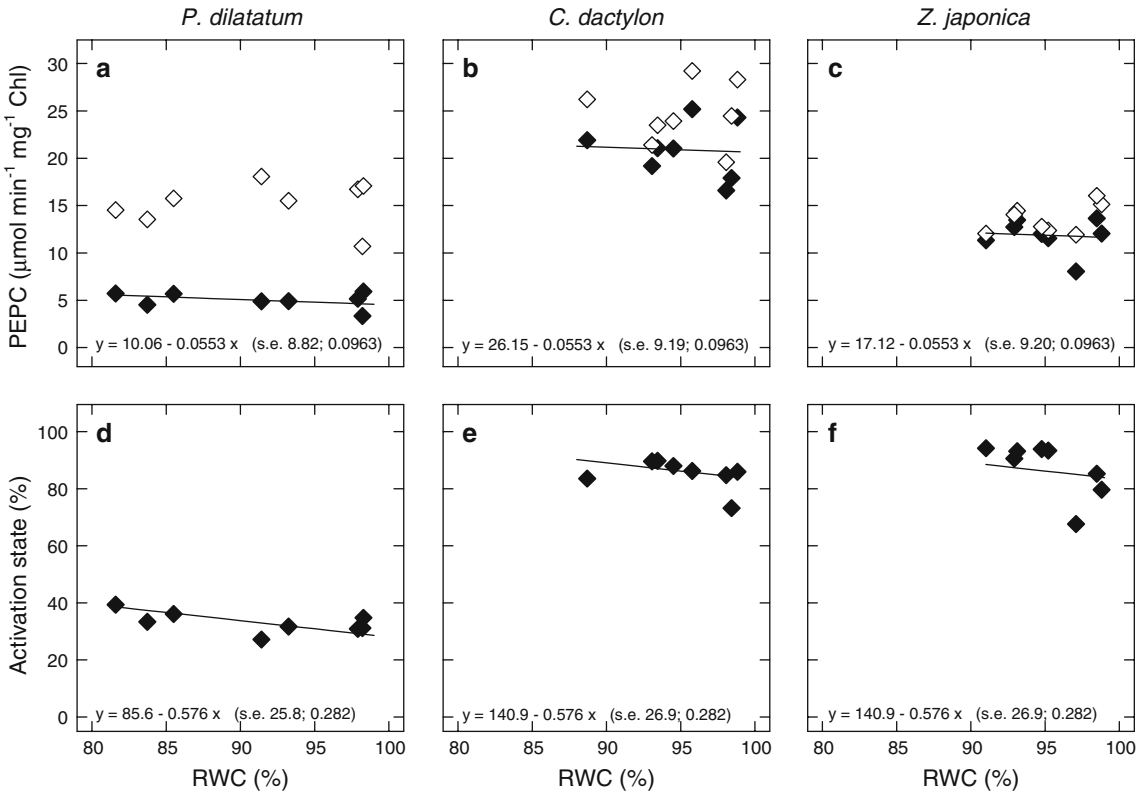

Fig. 1 (**a**–**c**) PEPC activities (µmol min^{-1} mg^{-1} Chl) and (**d**–**f**) activation state (%) as a function of the relative water content (RWC, %) in the leaves of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. Activity was measured under physiological ($V_{physiol}$; closed symbols) and optimal conditions (V_{max}; open symbols), and the activation state (**d**–**f**) was calculated as 100 × $V_{physiol}/V_{max}$. Each data point corresponds to one sample (with 8 samples/species). Regression lines were fitted when the RWC effect was significant ($P < 0.05$): $V_{physiol}$, $R^2 = 91.0\%$, $s^2 = 4.325$, df = 20; activation state, $R^2 = 94.7\%$, $s^2 = 37.1$, df = 21

(Fig. 1): the physiological activity of the enzyme increased slightly with decreasing RWC in all three species due to an increase in the activation state of the enzyme, and PEPC maximal activity was not affected by leaf dehydration. However, when leaf dehydration was rapidly induced by the addition of polyethylene glycol 4000 to the nutrient solution, PEPC activities were not affected in *P. dilatatum* and *C. dactylon* and decreased in *Z. japonica* only when RWC was below 70% (Carmo-Silva et al. 2004; 2007). The low activation state of PEPC in *P. dilatatum*, especially in comparison with the values obtained for *C. dactylon* and *Z. japonica* (Fig. 1), agrees with previous work with that species (Bernardes da Silva et al. 1995). For many plant species, the physiological concentration of PEP in illuminated leaves (ca. 2.5 mM) is assumed to be nearly saturating due to the low Km of PEPC for PEP, although this is dependent on the pH (O'Leary 1982). Echevarria et al. (1994) observed very little difference between the activity of immunopurified PEPC from *Sorghum* at pH 7.3 and at pH 8.0 with 2.5 mM PEP. In *C. dactylon*, PEPC activity was shown to saturate with 2 mM PEP at pH 7.2, and the physiological activity determined under such conditions represented 80% of the activity at pH 8.0 (Salahas and Gavalas 1997). In *P. dilatatum* a greater amount of PEP (ca. 10 mM) is required in order to saturate PEPC (Bernardes da Silva, unpublished data). The higher apparent Km of PEPC for PEP results in low physiological activity in proportion to the maximal activity in this NADP-ME species.

The drought-induced increase of PEPC physiological activity in all three grasses (Fig. 1) is consistent with increased phosphorylation of the enzyme, as indicated by the decreased sensitivity to the inhibitor L-malate (Fig. 2). In fully hydrated leaves, PEPC from *C. dactylon* was relatively less inhibited by L-malate than the enzyme from *P. dilatatum* and *Z. japonica*, suggesting a higher phosphorylation state in the former species (Fig. 2). The extent of inhibition of the enzyme from drought-stressed plants was similar for the three C_4 grasses. Even though aspartate was a weaker inhibitor of PEPC than L-malate, similar relative inhibition patterns were observed with the two C_4 acids in the two aspartate-forming species, *C. dactylon* and *Z. japonica*. The activation of PEPC by glucose-6-phosphate was greater in *P. dilatatum* than in *C. dactylon* and *Z. japonica*. Changes in L-malate and glucose-6-phosphate were previously observed in severely dehydrated leaves of

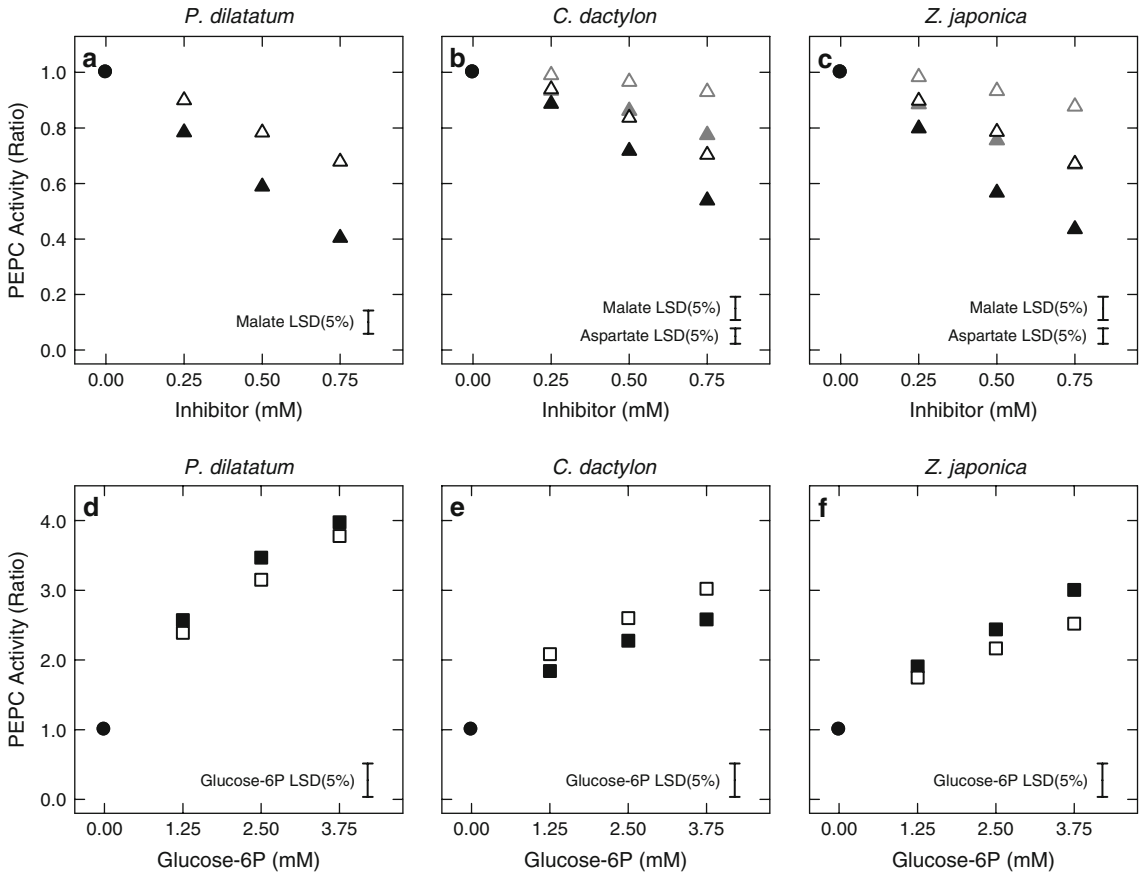

Fig. 2 Sensitivity of PEPC physiological activity ($V_{physiol}$; at pH 7.3) to the concentration of the inhibitor L-malate (**a**–**c**) and the activator glucose-6-phosphate (**d**–**f**) in the leaves from control (closed symbols) and non-watered (open symbols) plants of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. The effect of aspartate was also analysed on the two latter species. The ratio of the enzyme activity at each concentration of malate (black triangles), aspartate (grey triangles) and glucose-6-phosphate (black squares) relative to the activity in the absence of added effectors (closed circles) was calculated for each sample, and the mean values for each group of plants were analysed by the REML method. The overall least significant difference at the 5% level (LSD(5%)) is 0.084 for malate (48 df), 0.056 for aspartate (32 df) and 0.480 for glucose-6-phosphate (51 df)

Saccharum sp. (Du et al. 1998). It is plausible that changes in the concentrations of metabolite effectors in the dehydrated leaves may contribute to the regulation of the enzyme activity in vivo.

In *Flaveria bidentis* net CO_2 assimilation was not affected by a low phosphorylation of PEPC and raised the question of what may be the physiological role of the complexly regulated reversible phosphorylation of the enzyme (Furumoto et al. 2007). The increased phosphorylation state of PEPC in all three C_4 grasses under drought stress, and the consequent increase in activity, may be a strategy to maintain the rate of carboxylation as the concentration of CO_2 in the M cells decreases, as a result of stomatal closure (Carmo-Silva et al. 2008). Thus, the C_4 acids supplied to the BS cells could be adequate to maintain a high CO_2 concentration at the Rubisco site and limit the competing oxygenation of RuBP, and hence photorespiration, even under drought stress, as observed by Carmo-Silva et al. (2008).

PEPCK had appreciable activity in all three grass species (Fig. 3), but the activation state of the enzyme was much lower in *P. dilatatum* than in *C. dactylon* and *Z. japonica*. The activity of PEPCK at optimal concentrations of Mg^{2+} and Mn^{2+} was not affected by drought stress, and the activity of the enzyme at physiological concentrations of the divalent ions changed with leaf dehydration only in *C. dactylon*, decreasing by 8.5% when the RWC decreased to 90%. The activation state of both PEPC and PEPCK was low in *P. dilatatum* and high in the other two species. In *C. dactylon* an increase of PEPC activation state and a decrease of PEPCK activation state were observed under the drought conditions attained, whilst in *Z. japonica* the activation state of both enzymes increased with decreasing RWC. An effective coordination between the activation and phosphorylation states of PEPC and PEPCK was observed in the PEPCK species *Panicum maximum* (Bailey et al. 2007). In some C_4 grasses, PEPCK is regulated by phosphorylation/dephosphorylation (Walker and Leegood 1996), and

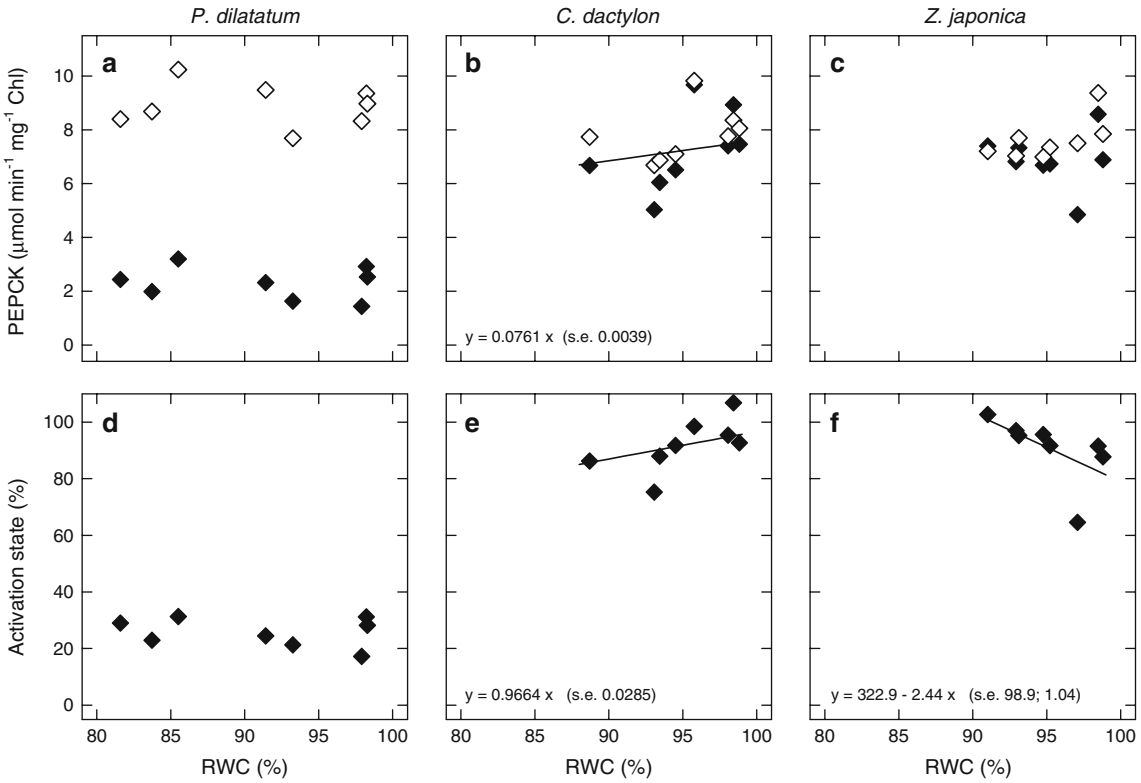

Fig. 3 (**a**–**c**) PEPCK activities (µmol min^{-1} mg^{-1} Chl) and (**d**–**f**) activation state (%) as a function of the relative water content (RWC, %) in the leaves of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. Activity was measured under conditions assumed to represent the closest to the physiological conditions (closed symbols) or at optimal concentrations of Mg^{2+} and Mn^{2+} (open symbols), and the activation state (**d**–**f**) was calculated as the ratio between the two multiplied by 100. Each data point corresponds to one sample (with 8 samples/species). Regression lines were fitted when the RWC effect was significant ($P < 0.05$): 'Physiological', $R^2 = 82.4\%$, $s^2 = 23.67$, df = 21; activation state, $R^2 = 94.5\%$, $s^2 = 58.8$, df = 20

therefore by the same type of covalent modification as PEPC, but activated by dephosphorylation (Walker et al. 1997; 2002). The enzyme from other species is not susceptible to modulation by phosphorylation but regulated by other mechanisms, possibly involving alterations in metabolite levels or ATP concentration (Walker et al. 1997; Leegood and Walker 1999).

NADP-ME activity decreased with leaf dehydration only in *P. dilatatum* (Fig. 4). The enzyme had no appreciable activity in *C. dactylon* and *Z. japonica*, consistent with the view that this enzyme has no important role in C_4 acid decarboxylation in the BS cells of these two species. A decrease in the activity of NADP-ME in *P. dilatatum* was also observed under rapidly induced leaf dehydration (Carmo-Silva et al. 2004) and may result from proteolysis or down-regulation under stress conditions.

The activity of NAD-ME was highest in *C. dactylon*, the species classified as belonging to this subtype. The enzyme activity decreased slightly with leaf dehydration in this species (Fig. 4) but was not affected by decreasing RWC in the leaves of *Z. japonica*. There was no appreciable activity in *P. dilatatum*, where the major decarboxylating enzyme is NADP-ME. A decrease in NAD-ME activity in *C. dactylon*, with no change in the enzyme activity in *Z. japonica*, was also found when drought conditions were rapidly imposed (Carmo-Silva et al. 2004). Leaf dehydration may cause decreased NAD-ME activity through a direct effect resulting in degradation or inactivation of the enzyme or through an indirect effect on the levels of adenylates or on the NADH/NAD^+ ratio (Leegood and Walker 1999). The NAD-ME from some but not all species is activated by ATP, whereas ADP and AMP invariably inhibit the enzyme (Furbank et al. 1991).

The activities of PEPC and the three C_4 acid decarboxylases were not affected by water deficit to an extent that would contribute to the limitation of photosynthesis. In a previous study, Carmo-Silva et al. (2008) observed decreased net CO_2 assimilation rates and stomatal closure in all three species under drought stress. Using average chlorophyll contents for each species to convert the data, the photosynthetic rates obtained at ambient air and under well-watered conditions (Carmo-Silva et al. 2008) would correspond to ca. 4.4 (*P. dilatatum*), 4.9 (*C. dactylon*) and 3.1 (*Z. japonica*) µmol min^{-1} mg^{-1} Chl. Thus, the results here presented reveal that the physiological activities of the C_4 enzymes involved in the carboxylation, and

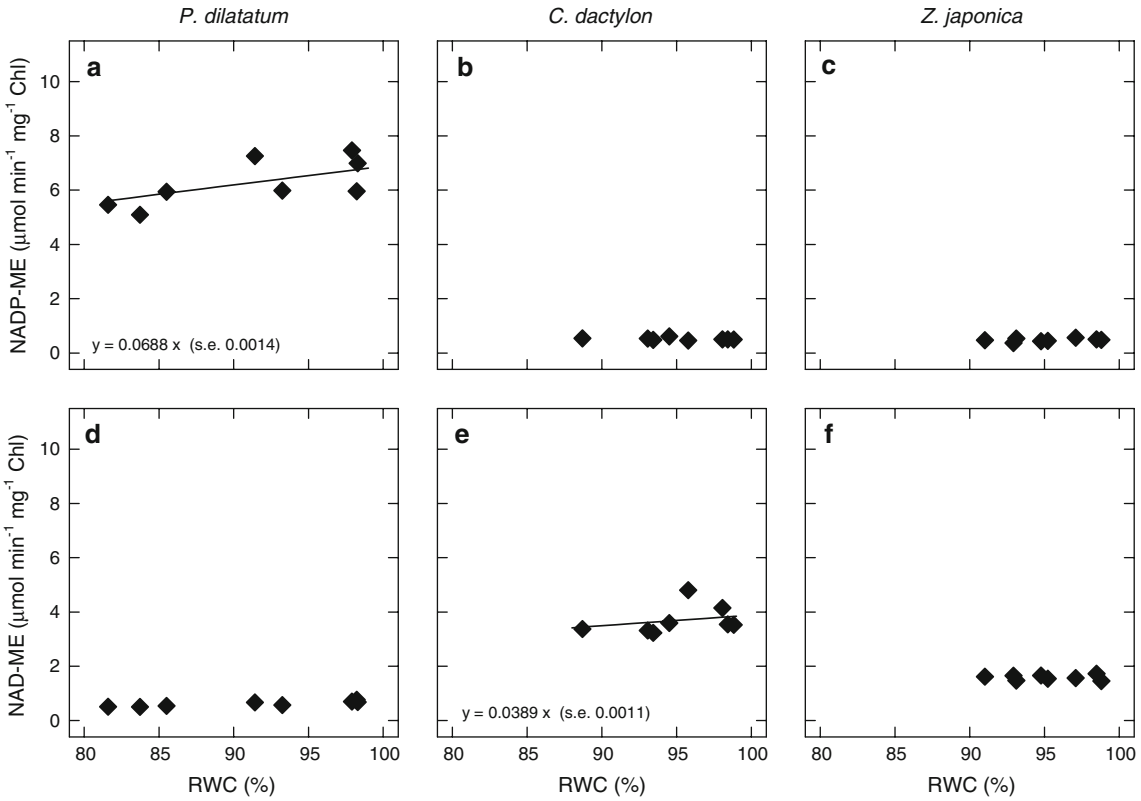

Fig. 4 Activities of (**a**–**c**) NADP-ME and (**d**–**f**) NAD-ME (µmol min^{-1} mg^{-1} Chl) as a function of the relative water content (RWC, %) in the leaves of *Paspalum dilatatum*, *Cynodon dactylon* and *Zoysia japonica*. Activities were measured under conditions assumed to represent the closest to the physiological state. Each data point corresponds to one sample (with 8 samples/species). Regression lines were fitted when the RWC effect was significant ($P < 0.05$): NADP-ME, $R^2 = 98.4\%$, $s^2 = 0.130$, df = 22; NAD-ME, $R^2 = 95.1\%$, $s^2 = 0.089$, df = 21

decarboxylation steps were adequate or considerably higher than the net CO_2 assimilation rates by the three grasses.

C_4 acid decarboxylases in *P. dilatatum*, *C. dactylon* and *Z. japonica*

Species belonging to the NADP-ME and NAD-ME subtypes of C_4 photosynthesis (Gutierrez et al. 1974; Hatch et al. 1975) have low activities of the other two C_4 acid decarboxylases. Conversely, in the PEPCK subtype, NAD-ME contributes considerably to the release of CO_2 in the BS cells and provides the required ATP for PEPCK activity (Kanai and Edwards 1999). By measuring simultaneously, in the same leaf extracts, the activities of the three C_4 acid decarboxylases under conditions that were assumed to be close to the physiological state, we observed the presence of PEPCK activity in all three species. While NAD-ME activity was present in *C. dactylon* and *Z. japonica* but was very low in *P. dilatatum*, NADP-ME was present in *P. dilatatum* but was very low in the other two grasses (Figs. 3 and 4). The relative activities of the C_4 acid decarboxylases in *P. dilatatum* agree with previous reports showing the presence of PEPCK in some C_4 grasses of the NADP-ME subtype, such as *Zea mays* and *Paspalum notatum* (Walker et al. 1997). Studies in *Zea mays* revealed that both PEPCK and NADP-ME contribute to the release of CO_2 in the BS cells of the species (Wingler et al. 1999). However, the presence of PEPCK in species belonging to the NADP-ME subtype is not ubiquitous, and neither protein nor mRNA of the enzyme was detected in *Sorghum* (Walker et al. 1997; Wyrich et al. 1998).

In species of the NAD-ME subtype, the activity of PEPCK has usually been found to be very low or negligible. Particularly in *C. dactylon*, PEPCK activity was found to be very low by Edwards et al. (1971), and the presence of the enzyme was not detected by Hatch and Kagawa (1974) and Prendergast et al. (1987). However, Fig. 3 shows high activities of PEPCK in *C. dactylon*, well above the activity found for NAD-ME (Fig. 4). Prendergast et al. (1987) suggested that some species might be intermediate biochemically, showing either predominance of NAD-ME or PEPCK activity. The same authors found masked activity of PEPCK in the C_4 grass *Tridens brasiliensis*, suggesting the presence of some compound in the leaves of this species that interfered with PEPCK detection

and measurement by the methods then used. Therefore, special care was taken to include the appropriate components in the extraction medium to maximise the extraction and recovery of the enzyme activities. The methods adopted in the present study were similar to those recently reported by other authors (Bailey et al. 2007; Marshall et al. 2007; Sudderth et al. 2007), and the values obtained for the activities of the three C_4 acid decarboxylases are in the same range as previously reported for grass species (Gutierrez et al. 1974; Hatch et al. 1975, 1982; Prendergast et al. 1987; Ueno and Sentoku 2006).

The decarboxylation by PEPCK can be viewed as a relatively simple addition to, or variant of, the NAD-ME pathway (Kellogg 1999). The high activities of PEPCK in *P. dilatatum* and *C. dactylon* suggest that this enzyme may act as a supplementary decarboxylating enzyme to NADP-ME and NAD-ME (Kellogg 1999; Lea et al. 2001). However, PEPCK is also known to have non-photosynthetic functions in amino acid, organic acid, sugar, lipid and secondary metabolism (Leegood and Walker 2003). The presence of high PEPCK activity needs to be further investigated and complemented with studies to characterise and quantify the enzyme type present in each of the three species. The functional significance of high PEPCK activity in the leaves of the three species studied and in other C_4 grasses must be clarified.

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