Photosynthetic enzymes of the C_4 grass *Setaria sphacelata* under water stress: a comparison between rapidly and slowly imposed water deficit

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Abstract

Two stress imposing systems were used: a rapid stress developed by allowing excised leaves to loose water by transpiration, and a slow stress developed by withholding watering of potted plants. Carboxylating enzymes reacted differently on both types of stress. Rapid stress increased ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) activation, but both activities (initial and total) showed little variation with stress. Under slow stress the activation did not change, although both activities decreased much under stress. Phospho*enol*pyruvate carboxylase (PEPC) showed a deep decrease of activity under rapid stress, nevertheless, a certain recovery was found under extreme stress. On the other hand, under slow stress the activity of PEPC showed a linear increase with decreasing relative water content. The ratio between physiological and maximal activity increased slightly under both types of stress. The activity of malic enzyme did not change under rapid stress, and decreased linearly under slow stress.

Additional key words: NADP-malic enzyme; phospho*enol*pyruvate carboxylase; ribulose-1,5-bisphosphate carboxylase/oxygenase.

Introduction

The most characteristic effects of water stress on plant growth are the decreases of leaf expansion and photosynthetic rate. The decrease in photosynthesis is certainly due to a lower chloroplast CO_2 concentration, as a consequence of stomata closure (*e.g.* Medrano *et al*. 2002), and to alterations at the carbon fixation level, namely as concerns ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO, E.C. 4.1.1.39) and phospho*enol*pyruvate carboxylase (PEPC, E.C. 4.1.1.31). In C_4 plants, *e.g.* in *Setaria sphacelata* var. *splendida* (Stapf) Clayton, PEPC pre-fixes atmospheric carbon. The de-carboxylating enzyme of this photosynthetic sub-type, NADP-dependent malic enzyme (NADP-ME), may also be important in this process, as the adequate operation of C_4 photosynthesis under stress requires a large intercellular metabolic coordination, including the maintenance of large fluxes of metabolites between the two cell types (Foyer *et al.* 2002). The effects of water stress on the activity of photosynthetic enzymes are still a matter of strong controversy: some authors report notorious decline of activity and others claim for no effects. This controversy is certainly due, at least in part, to the fact that very different stress situations are used in respective experiments and some of these differences are not properly taken into account. Among these, the rate of stress development may influence the results (Ögren 1990), but systematic studies on the influence of this factor are scarce, and, as far as we know, absent in C_4 plants. Therefore, the aim of this study was to compare the effects on photosynthetic enzymes of rapidly and slowly imposed water stress.

Materials and methods

Plants: *Setaria sphacelata* var. *splendida* plants (Mozambique's Umbeluzi Valley ecotype) were grown in 18 pots, one plant *per* pot, in a mixture of peat and soil (3 : 7), under a photosynthetic photon flux density (PPFD) of approximately 400 μmol m^{-2} s^{-1}, a photoperiod of 16 h, a day/night temperature of 25/18 °C, and a relative humidity of 50–70 %. Plants were abundantly watered 3 times a week until they were 2 months old, then the sampling started. Regression analysis was performed between all enzymatic parameters and relative water content (RWC). Linear and quadratic models were applied, and the most significant was chosen through an F-test, as in Sokal and Rohlf (1995).

Received 28 July 2003, *accepted* 15 January 2004.
Acknowledgements: We are indebted to Dr. Anabela Bernardes da Silva for assistance in PEPC determination and to Eng. D. Coelho Rebelo for generously providing *S. sphacelata* plants.

Rapidly induced water deficit experiments: Six leaf fragments of 2 cm^2 and one of 4 cm^2 were cut from the middle zone of young fully-developed leaves sampled from plants in 6 pots. Excised fragments were dehydrated by exposure to the atmosphere of the growth chamber. A water deficit of 50 % was reached in approximately 3 h. The biggest fragment was periodically weighed and was used as an indicator of RWC of the other fragments. These were sequentially frozen in liquid nitrogen and a sequence of leaf samples progressively more dehydrated was obtained.

Slowly induced water deficit experiments: Water stress was induced by total withdrawal of watering 6 pots, starting when plants were 2 months old. The remaining 6 pots were kept watered and were used as control. On average, leaves attained 50 % RWC after 45 d of the stress. Leaf segments (around 2 cm^2) were removed during the period of stress from plants submitted to water withdrawal and immediately frozen in liquid nitrogen.

Enzyme extraction: The three enzymes under study were extracted simultaneously from the frozen fragments, which were kept in a freezer at –70 °C. The extraction medium was adapted from Edwards *et al.* (1988) and was composed of 50 mM HEPES, pH 7.4, 5 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 1 mM pyruvate, and 20 % glycerol (Jiao *et al.* 1991), 5 % polyvinyl pyrrolidone (m/v), and 2 % *Polyclar AT* (m/v). Approximately 2 cm^2 of leaf were ground in a chilled mortar and pestle with 0.2 cm^3 of the extraction medium for 30–40 s, and 1 cm^3 of the medium was added afterwards. An aliquot of 50 mm^3 was removed for later determination of chlorophyll content, and the remaining extract was centrifuged at 15 800×*g* for 30 s (*5415 C, Eppendorf*, Hamburg, Germany). The supernatant was recovered and stored on ice until enzymatic assays. In rapid stress experiments, the activities of the three enzymes were immediately determined. In slow stress experiments, the activities of PEPC and NADP-ME were immediately determined and the remaining extract was frozen at –70 °C, and the activity of RuBPCO was measured the following day.

Enzyme activities: RuBPCO activity was determined through the incorporation of labelled CO_2 according to Perchorowicz *et al.* (1982). The assay medium was composed of 100 mM HEPES, pH 7.7, 20 mM KCl, 30 mM $MgCl_2$, 1 mM DTT, and 12 mM $NaH^{14}CO_3$ (3.7×10^{10} Bq mol^{-1}). For the determination of total activity, 20 mm^3 of the extract were added to 460 mm^3 of the assay medium. After 5 min of incubation, 20 mm^3 of 15 mM RuBP was added. The reaction proceeded for 30 s and then was stopped with 500 mm^3 of 1 M HCl. For the determination of the initial activity, RuBP was added before the extract, and the remaining procedure was similar. The per cent of activation was the ratio between initial and total activities × 100. The reaction samples were afterwards transferred to an oven at 80 °C where they remained until total drying. The residue was dissolved in 2 cm^3 of ultra-pure water and shaken in a vortex. 8 cm^3 of a scintillation cocktail was added and the samples were measured in a liquid scintillation spectrometer (*LS 9800, Beckman Instruments,* Fullerton, California, USA).

NADP-ME maximal activity was determined according to Edwards *et al.* (1982) as described in Simon (1987), with the following modifications: The assay medium was composed of 50 mM HEPES, pH 8.0, 5 mM DTT, 75 mM $MgCl_2$, and 1.25 mM NADP. 25 mm^3 of the extract was incubated with this mixture for 5 min and after that the reaction was started with 12.5 mM malate.

PEPC maximal activity was determined spectrophotometrically by a modified method of Donkin and Martin (1980), in an assay medium composed of 25 mM HEPES, pH 8.0, 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 0.1 mM NADH, 12.5 mM PEP, and 8 units of malate dehydrogenase (MDH). The reaction was initiated with 100 mm^3 of the extract. The activity under physiological conditions was obtained with 25 mM HEPES, pH 7.2 and 2.5 mM PEP, keeping all the other conditions unchanged.

The amount of RuBPCO protein was determined by ^{14}CABP binding according to Parry *et al*. (1993).

Soluble protein content present in enzymatic extracts was determined according to Bradford (1976).

Results

RuBPCO total activity behaved differently when two different stress situations were imposed: at rapid stress an unchangeable total activity with decreasing RWC was found (Fig. 1*A*), while in the slowly induced stress for the same range of water deficit a notorious decline was found (Fig. 1*B*). The difference was even higher for an initial activity, since opposite responses were observed. In the rapidly stressed samples, the initial activity increased with the decrease of RWC (Fig. 1*A*), whereas in the slowly stressed samples, a decrease was found (Fig. 1*B*). Under the rapid stress, the per cent activation of RuBPCO increased with decreasing RWC, while in slow stress it was maintained (Fig. 1*C*). Under the rapid stress, an increase in RuBPCO amount as a % of total soluble protein was found with decreasing RWC, but under the slow stress no correlation was found between the two parameters (Fig. 2*A*). Similar results were obtained when RuBPCO amount was expressed on a dry mass basis (Fig. 2*B*). Total specific activity decreased with the decrease in RWC under both the rapid and slow stresses. Nonetheless, the decrease was more accentuated in the latter case (Fig. 2*C*). Initial specific activity did not change with decreasing RWC under the rapid stress, but decreased sharply under the slow stress (Fig. 2*D*). Under

the rapid stress both the maximal and physiological activities of PEPC sharply decreased with decreasing RWC, with a notorious recovery under extreme values of stress (Fig. 3*A*). Under the slow stress a different behaviour was found, with an increase of both activities as the RWC decreased (Fig. 3*B*). The ratio between physiological and maximal PEPC activities increased only slightly with decreasing RWC in both types of stress (Fig. 3*C*). The activity of NADP-ME did not correlate with RWC under the rapid stress and decreased linearly as RWC decreased under the slow stress (Fig. 4).

Discussion

The imposition of a rapid water deficit seems to trigger the response that leads to an increase of the *de novo* synthesis of RuBPCO, as shown by its increase as a % of the total soluble protein and per dry mass (Fig. 2*A*). An increase of RuBPCO protein content under water stress has not been previously reported, probably due to the absence of very rapid short-term dehydration experiments. However, when a slow stress was imposed this response was not observed, degradation trends were not found, and no correlation between the amount of RuBPCO and RWC was detected. This is in accordance with the reported RuBPCO holoenzyme relative stability under water stress, with a half-life of several days (Webber *et al.* 1994), albeit in *Arabidopsis* (Williams *et al*. 1994) and rice (Vu *et al*. 1999) drought lead to a rapid decrease in the abundance of RuBPCO small subunit transcripts, which may indicate decreased synthesis. In spite of the absence of changes in its amount (Fig. 2*A,B*), initial and total activities of RuBPCO decreased under slow stress (Fig. 1*B*), probably as the result of a decrease in initial and total specific activities of the enzyme (Fig. 2*D*). This decrease may be related to the binding of day-time and night inhibitors, as discussed by Parry *et al*. (2002). This is further supported by the fact that the initial and total activities decreased proportionally, maintaining then the % activation. However, under rapid stress the initial specific activity (Fig. 2*C*) did not correlate with RWC and the total specific activity decreased, suggesting that the mechanisms leading to the changes of specific activities operating under rapid and slow stress conditions are different. There is no evidence of accumulation of an inhibitor under the rapid stress, and the increase in the % activation (Fig. 1*C*) as a consequence of an increase of the initial activity suggests a higher carbamylation level of RuBPCO *in vivo*. In another NADP-ME C_4 grass, *Paspalum dilatatum*, a decrease of the % activation was found under similar rapid water stress conditions, due to simultaneous increase of the total activity and decrease of the initial activity (Arrabaça *et al*. 2001).

PEPC activity decreased sharply under the rapid stress, showing a notorious increase under extreme stress values (Fig. 3*A*). Ogawa *et al*. (1997) proposed that the activity of PEPC could increase under water stress due to the stabilizing effect of the increased concentration of osmoprotecting agents. A different behaviour was found under the slow stress, where both activities increased with increasing water deficit. Saccardy *et al*. (1996) did not find any changes in maize PEPC activity, neither under rapid nor under slow water stresses. In *P. dilatatum* Bernardes da Silva *et al*. (2001) found an increase of PEPC activity both under rapid and slow water stress

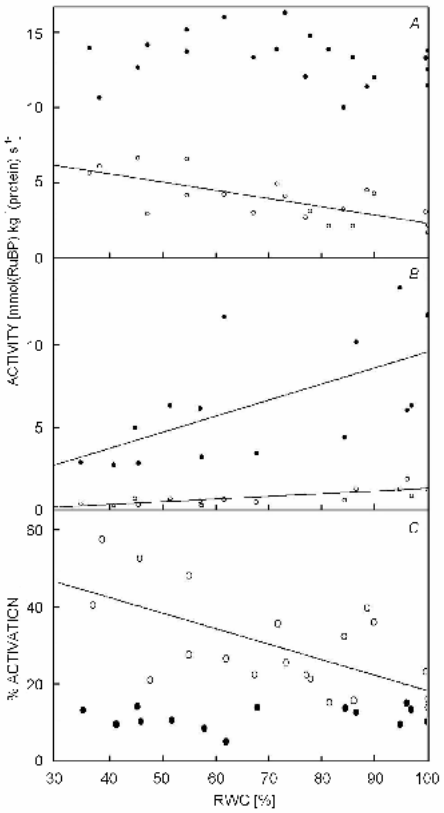

Fig. 1. Total (●) and initial (○) RuBPCO activities and % of RuBPCO activation (*C*) in rapidly (*A*) or slowly (*B*) stressed leaves, expressed per soluble protein, as a function of relative water content (RWC). Whole samples were taken from 4 different leaves and each value is the mean of 3 replicates of activities measured in the same crude extract. (*A*): No significant correlation was found between the total activity under rapid stress (●) and RWC (r = 0.222, p>0.1). The line adjusted to initial activity under slow stress (○) holds the equation: y = 7.838 – 0.055 x (r = 0.732, p<0.001). (*B*): The line adjusted to total activity under slow stress (○) holds the equation: y = –0.261 + 0.099 x (r = 0.618, p<0.02). The line adjusted to initial activity under slow stress (○) holds the following equation: y = –0.347 + 0.916 x (r = 0.792, p<0.001). (*C*): The line adjusted to rapid stress (○) holds the following equation: y = 58.510 – 0.401 x (r = 0.646, p<0.005). No significant correlation was found between the % de-activation of RuBPCO under slow stress (●) and RWC (r = 0.618, p<0.02). Values were calculated from Fig. 1*A*,*B*.

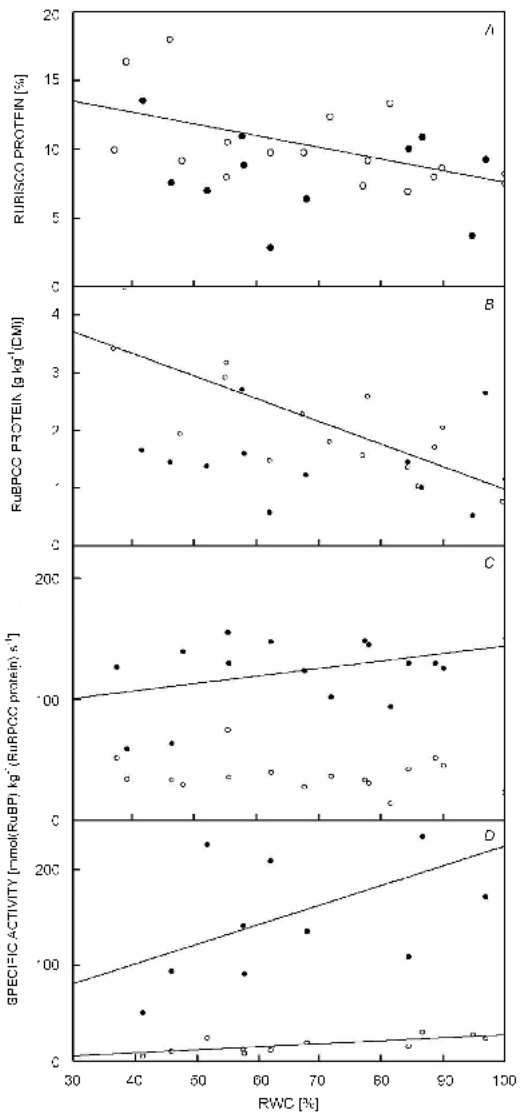

Fig. 2. Effects of water stress on RuBPCO protein and specific activity. Means of 3 replicates, measured in some of the extracts used for the measurement of enzymatic activities. (*A*): Per cent RuBPCO protein in total protein (○ = rapid stress, ● = slow stress) as function of relative water content (RWC). The line adjusted to rapid stress (○) holds the equation: y = 16.070 – 0.085 x (r = 0.551, p<0.05). No significant correlation was found between the % of RuBPCO in total protein under slow stress (●) and RWC (r = 0.447, p>0.10). (*B*): Amount of RuBPCO protein expressed per dry mass (○ = rapid stress, ● = slow stress) as a function of RWC. The line adjusted to rapid stress (○) holds the equation: y = 4.898 – 0.039 x (r = 0.804, p<0.001). No significant correlation was found between the % of RuBPC in total protein under slow stress (λ) and RWC (r = 0.447, p>0.1). (*C*): Total (●) and initial (○) specific activity of RuBPCO as a function of RWC in rapidly stressed leaves. The line adjusted to total specific activity holds the equation: y = 90.919 + 0.678 x (r = 0.431, p<0.1). No significant correlation was found between the initial specific activity under rapid stress (○) and RWC (r = 0.298, p>0.1). (*D*): Total (●) and initial (○) specific activity of RuBPCO as a function of RWC in slowly stressed leaves. The line adjusted to total specific activity (●) holds the equation: y = 19. 044 + 2.050 x (r = 0.567, p<0.1). The line adjusted to initial specific activity under slow stress (○) holds the following equation: y = –4.514 + 0.314 x (r = 0.733, p<0.02).

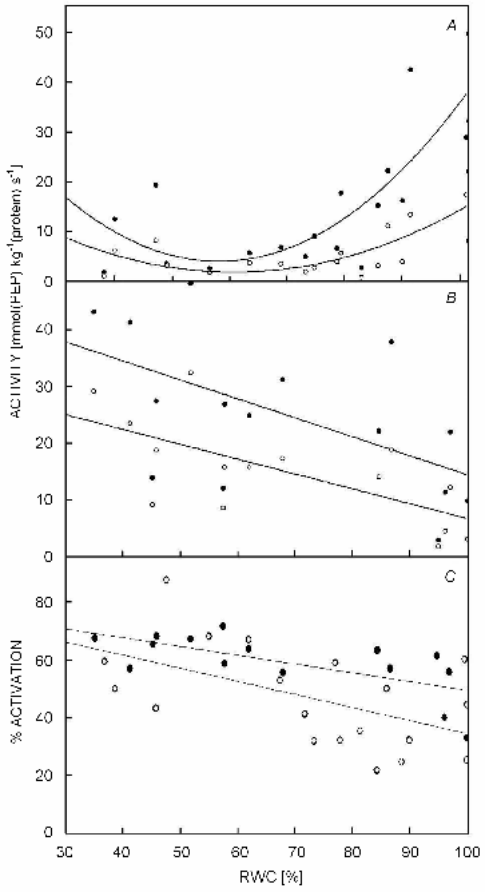

Fig. 3. Effects of water stress on PEPC activity. (*A*) Maximal activity (●) and physiological activity (○) of PEPC, expressed by soluble protein, as a function of relative water content (RWC) in rapidly stressed leaves. Each point corresponds to one measurement of activity. The line adjusted to maximal activity holds the equation: y = 62.761 – 2.069 x + 0.018 x^2 (r = 0.823, p<0.001). The line adjusted to physiological activity holds the equation: y = 30.667 – 0.969 x + 0.008 x^2 (r = 0.765, p<0.001). (*B*): Maximal activity (●) and physiological activity (○) of PEPC, expressed by soluble protein, as a function of RWC in slowly stressed leaves. Each point corresponds to one measurement of activity. The line adjusted to maximal activity holds the equation: y = 47.781 – 0.336 x (r = 0.567, p<0.05). The line adjusted to physiological activity holds the equation: y = 33.025 – 0.263 x (r = 0.672, p<0.01). (*C*): Per cent activation measured by the ratio between physiological and maximal activity of PEPC (○ = rapid stress, ● = slow stress) as a function of RWC. Values obtained from Fig. 3*A,B*. The line adjusted to data from rapid stress holds the equation: y = 79.720 – 0.453 x (r = 0.530, p<0.02). The line adjusted to data from slow stress holds the following equation: y = 79.770 – 0.304 x (r = 0.661, p<0.01).

conditions, *i.e.* the dramatic initial decrease of activities found in *S. sphacelata* was absent. The notorious recovery of these activities found under intense rapid stress suggests that the plant responds to the initial decrease with later increase, similar to the increase found in slow stress experiments (Fig. 3*B*). This increase in PEPC activities found under severe stress may be due to activation by G_6P that accumulates under stress conditions (Willeford and Wedding 1992), the effect being more evident under slow stress. The ratio between physiological and maximal activities increased under both types of stress, suggesting that the phosphorylation/de-phosphorylation processes and the regulation by metabolites may play a role in the variation of activities under stress, but probably the processes related to the oligomerization state and/or the degradation and synthesis of the enzyme are more relevant (Fig. 3*C*). The linear decrease of the activity of NADP-ME found under slow stress might be dependent on global senescence decay, and not on a specific regulatory process (Fig. 4).

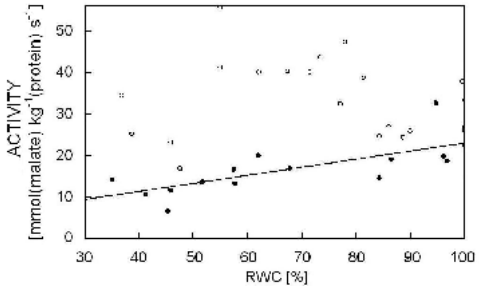

Fig. 4. Malic enzyme activity expressed per soluble protein, as a function of RWC (○ = rapid stress, ● = slow stress). Each point corresponds to one measurement of activity. No significant correlation was found between the activity under rapid stress and RWC ($r = 0.081$, $p>0.10$). The line adjusted to data from slow stress holds the equation: $y = 3.514 + 0.193\ x$ ($r = 0.732$, $p<0.005$).

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