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EFFECTS OF SODIUM CHLORIDE STRESS ON PHOSPHOENOLPYRUVATE CARBOXYLASE, NADP-MALIC ENZYME AND RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE IN SHOOTS OF PEARL MILLET

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Key Word Index—*Pennisetum* sp.; Gramineae; pearl millet; C_4 plants; shoots; phosphoenolpyruvate carboxylate; NADP-malic enzyme; ribulose-1,5-bisphosphate carboxylase; apparent specific activity; salt stress.

Abstract—The effects of treatment of seedlings with sodium chloride at 25 or 75 mM on phosphoenolpyruvate carboxylase (PEPc), NADP malic enzyme (NADP-ME) and ribulose-1,5-bisphosphate carboxylase (RuBPc) was studied in shoots of four Pearl millet genotypes. The results of this study reveal that the response of PEPc and NADP-ME to the mild treatment with 25 mM sodium chloride depends on the tolerance of seedlings to the salt. The specific activities of these enzymes were enhanced in Droô Zarzis, a salt-tolerant genotype, and decreased in *Pennisetum violaceum*, the most sensitive one. These results support the hypothesis that the specific activities of PEPc and NADP-ME may be effective markers of salt tolerance in the C_4 plant, Pearl millet.

INTRODUCTION

It was previously reported that the *in vitro* activities of two key enzymes of the C_4 cycle of photosynthesis, phosphoenolpyruvate carboxylase (PEPc) and NADP-malic enzyme (NADP-ME) from shoots of four *Pennisetum* genotype seedlings (*P. typhoides* cv. Droô Zarzis, *P. mollissimum*, *P. americanum* cv. 23 DB and *P. violaceum*) were affected by treatment of the seedlings with sodium chloride [1]. Salt stress stimulated PEPc activity of the four Pearl millets, NADP-ME activity of the most tolerant one, Droô Zarzis, but reduced the NADP-ME of *P. violaceum*, the least tolerant. *P. mollissimum* and 23 DB showed an intermediate behaviour. These observations suggested that the salt tolerance of Droô Zarzis could be explained by stimulation at the same time of carbon dioxide fixation by photosynthetic PEP carboxylation and carbon dioxide release from malate in the bundle sheath chloroplasts. On the other hand, sodium chloride sensitivity of the non-tolerant *P. violaceum* could be explained by shortage of malate decarboxylation in the bundle sheath chloroplasts.

The appearance of a new isoenzyme of PEPc in leaves of *Mesembryanthemum crystallinum* [2] and the decrease of PEPc activity in leaves of *Pennisetum* [3] were shown to be concomitant with the ionic imbalance resulting from sodium chloride treatment. In *Zea mays*, *Chloris gayana* [4] and wheat [5] the ratio of ribulose-1,5-bisphosphate carboxylase (RuBPc) to PEPc decreased with increasing sodium chloride in the nutrient medium. Other stresses such as light or phosphate deficiency were reported to stimulate the synthesis of the enzymes PEPc and NADP-ME [6–9].

The purpose of the research reported here was to investigate whether RuBPc was affected by sodium chloride treatment like PEPc in Pearl millets and to determine whether the changes in enzyme activities observed in salt-stressed *Pennisetum* were associated with changes in the amounts of PEPc, NADP-ME and RuBPc enzyme proteins. Rocket immunoelectrophoresis was used to determine the amount of enzyme proteins of 18- or 26-day-old wild (*P. mollissimum* and *P. violaceum*) and cultivated genotypes (Droô Zarzis and 23 DB), treated with 0, 25 or 75 mM sodium chloride.

RESULTS AND DISCUSSION

The response to sodium chloride stress was estimated by the fresh weight and soluble protein per seedling in the shoots of 18-, 22- and 26-day-old seedlings treated at the age of 8 days by addition of 0, 25 or 75 mM sodium chloride in the nutrient solution. Without any sodium chloride added to the medium (control seedling), the cultivated genotypes, i.e. Droô Zarzis and 23 DB, showed a slower fresh weight production (Figs 1a and 1c) than the wild genotypes *P. mollissimum* and *P. violaceum* (Figs 1b and 1d). No significant difference in soluble protein per seedling between the wild and cultivated genotypes was observed (Figs 1e–1h).

With 25 mM sodium chloride, the fresh weight yield was highly stimulated in Droô Zarzis. It was slightly reduced in *P. mollissimum* and *P. violaceum* and was not significantly changed in 23 DB. Upon addition of 75 mM sodium chloride, the fresh weights of all the genotypes were reduced. *P. violaceum* was the most affected, with a dramatic decrease in growth. Both wild genotypes were more sensitive to sodium chloride than the cultivated species. The halophytic character of Droô Zarzis was shown by the enhancement of fresh weight yield in the

Table 1. Effect of sodium chloride in the nutrient solution on activity, amount and apparent specific activity (activity/amount) of PEPc, NADP-ME and RuBPc in shoots of Pearl millet. Activity and relative amount of Pearl millet grown without sodium chloride were taken as 100

Genotype	NaCl treatment (mM)	PEP carboxylase			NADP-ME			RuBP carboxylase		
		Activity	Amount	Activity/ amount	Activity	Amount	Activity/ amount	Activity	Amount	Activity/ amount
P. typhoides cv. Droô Zarzis	0	100 (3.2)*	100†	1	100 (2.6)	100	1	100 (0.70)	100	1
	25	119	81	1.47	81	58	1.39			
	75	133	130	1.02	150	167	0.89	135	127	1.08
P. mollissimum	0	100 (2.7)	100	1	100 (2.0)	100	1			
	25	152	98	1.55	119	88	1.65			
	75	205	163	1.26	145	180	0.80			
P. americanum cv. 23 DB	0	100 (4.8)	100	1	100 (3.9)	100	1			
	25	97	98	0.99	97	104	0.93			
	75	124	125	0.99	120	169	0.71			
P. violaceum	0	100 (6.9)	100	1	100	100	1	100 (0.79)	100	1
	25	72	153	0.47	62	87	0.70			
	75	172	173	0.99	100	153	0.39	118	125	0.94

*Values in parentheses are enzyme activities in μmol NADH oxid/min/mg protein for PEPc; μmol NADP red/min/mg protein for NADP-ME; μmol CO_2/min/mg protein for RuBPc.
Amount 100 = arbitrary unit = height of the rocket.

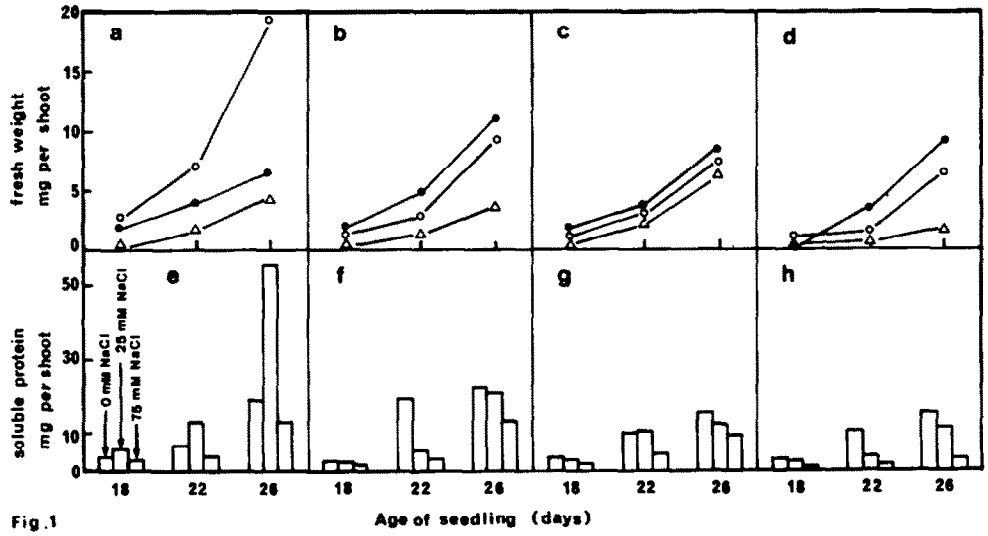

Fig. 1. Influence of sodium chloride in the nutrient solution on the growth and protein content of shoots of 18-, 22- and 26-day-old Pearl millet. (●) 0 mM NaCl; (○) 25 mM NaCl; (△) 75 mM NaCl. (a, e) *P. typhoides* Droô Zarzis; (b, f) *P. mollissimum*; (c, g) *P. americanum* 23 DB; (d, h) *P. violaceum*. Each value is the mean of three measurements. Maximum s.d. was 5%.

presence of 25 mM sodium chloride. Variations of soluble proteins per plant followed fresh weight production in all genotypes. Protein content per unit fresh weight was not affected by sodium chloride treatment. A similar observation on leaves of barley seedlings was reported by Aslam *et al.* [10].

Immunological quantification of enzymes

After treatment with 25 mM sodium chloride the variations in the amounts of PEPc and NADP-ME were not always similar to the variations in enzyme activities (Table 1). In Droô Zarzis and *P. mollissimum*, the amounts of the two protein enzymes were negatively affected or unchanged. Consequently, the apparent specific activities increased. In 23 DB, no variation in amount or activity of the two enzymes was observed. The apparent specific activities remained constant. In *P. violaceum*, the amount of PEPc increased and in contrast, the amount of NADP-ME decreased, but the apparent specific activities of the two enzymes declined.

When treated with 75 mM sodium chloride concomit-

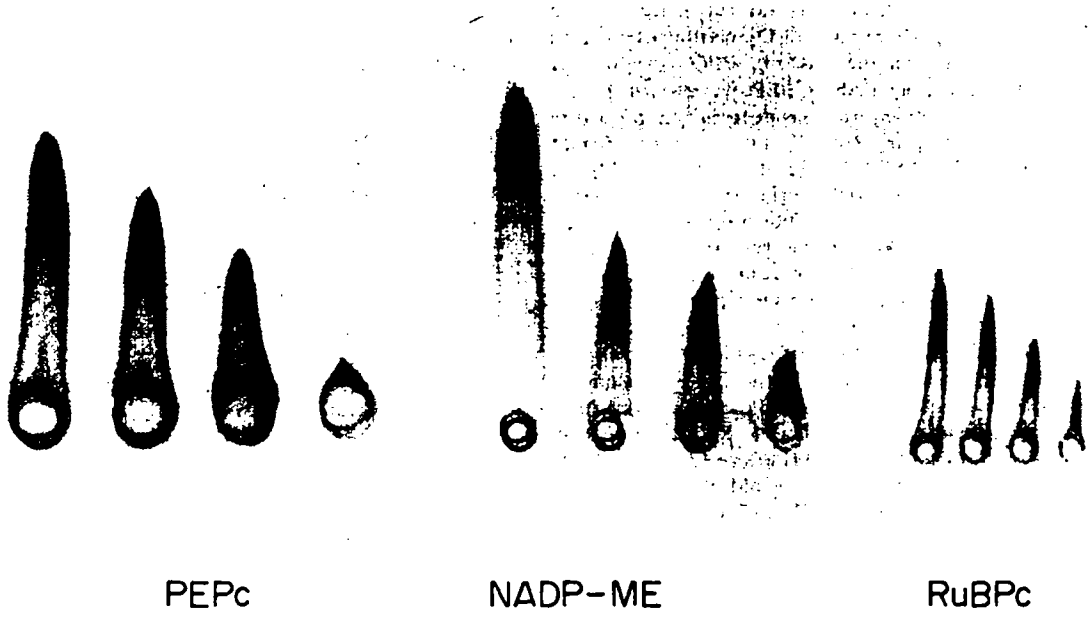

Fig. 2. Rocket immunoelectrophoresis of phosphoenolpyruvate carboxylase (PEPc), NADP-malic enzyme (NADP-ME) and ribulose-1,5-bisphosphate carboxylase (RuBPc) from shoots of control *Pennisetum* seedlings (no sodium chloride added to the nutrient solution). The amount of each protein enzyme in the extracts of salt-treated seedlings was within this range of rocket height.

ant increases in the amounts and activities of PEPc and RuBPc were observed. As a consequence, the apparent specific activities were not affected. The amounts of NADP-ME increased more than the activities of the same and the apparent specific activity decreased in all genotypes.

The drastic treatment with 75 mM sodium chloride which altered the growth of the four Pearl millets resulted in a better representation of the three main enzymes of photosynthetic carbon dioxide fixation among the pool of soluble proteins without affecting their specific activities.

In conclusion, the results of this study reveal that the response of PEPc and NADP-ME to the mild treatment with 25 mM sodium chloride depends on the tolerance of the seedlings to the salt. On the basis of the specific activities of these two enzymes, three kinds of response were observed. In all cases the changes in specific activity of PEPc and NADP-ME were similar. They were enhanced in Droô Zarzis and *P. mollissimum*, unaffected in *P. americanum* 23 DB and decreased in *P. violaceum*. This classification fits the classification of decreasing salt tolerance estimated by fresh weight yield, which showed Droô Zarzis to be very tolerant, *P. mollissimum* to be less tolerant, *P. americanum* to be insensitive and *P. violaceum* to be very sensitive [1]. These results support the hypothesis that the specific activities of PEPc and NADP-ME may be effective markers of salt tolerance in the C_4 plant, Pearl millet.

EXPERIMENTAL

Plant material. Seeds of *P. americanum* (L.) Leeke cv. 23 DB, *P. violaceum* (L). Rich were provided by ORSTOM (Office de Recherches Scientifiques dés Territoires d'Outre-Mer). Bondy, France. Seeds of *P. typhoides* cv. Droô Zarzis and *P. mollissimum* were provided by the Laboratoire de Génétique et Physiologie du Développement des Plantes, CNRS, Gif sur Yvette, France.

Seeds were sown in pots filled with sand. Seedlings were grown in a controlled environment chamber. In order to minimize evaporation, the sand surface was covered with a layer of plastic film.

The seedlings were watered daily with deionized water. Light was provided by Osram lamps giving 250–270 W m^2 for 14 hr a day. Day and night temps. were 28° and 23°, respectively. Humidity was 70%. Salinity stress was imposed upon 8-day-old plants. Seedlings were watered every other day with a nutrient soln described by Blondon [11] containing 0 (control), 25 mM or 75 mM NaCl. It is well known that Na^+ in trace amounts is absolutely required by plants with photosynthetic carbon fixation via the C_4 pathway [1]. In the present study Na^+ is provided to the medium by impurities of the different other salts.

Preparation of shoot tissue extract for enzyme assay. Shoots from each genotype were frozen and ground in a prechilled mortar with a pestle. The resulting powder was then homogenized in ice cold extraction medium. The brei was filtered through a 36 μm nylon gauze and centrifuged for 15 min at 15000 *g* in a refrigerated centrifuge. The supernatant was assayed for enzyme activities. The extraction medium for RuBPc contained 100 mM Tris–HCl (pH 8), 20 mM KCl, 2.5 mM EDTA. 70 μM PMSF and 2% Polyclar AT(W/V). For PEPc and NADP-ME, the extraction medium included 50 mM Tricine (pH 8) and 5 mM DTT. All operations were conducted at 4°.

Assay of enzyme activities. RuBPc was assayed following the substrate-dependent $H^{14}CO_3$ incorporation into acid-stable products. The assay medium contained 40 mM Tris–HCl (pH 8), 40 mM $MgCl_2$, 4 mM DTT, 15 mM $NaH^{14}CO_3$ (2 μCi/μmol) plus enzyme extract in a total vol. of 62.5 μl. After 10 min of preincubation at 30°, 0.6 mM RuBP was added to start the reaction. The reaction was stopped after 60 sec by the addition of 25 μl 6 M HCl. After evaporation of the mixture to dryness, the

radioactivity in acid-stable products was determined by liquid scintillation counting. The released $^{14}CO_2$ was trapped in dioxan soln containing naphthalene (0.47 M), PPO (18 mM) and POPOP (0.51 mM) as a scintillant. PEPc was assayed spectrophotometrically by coupling oxaloacetate formation with malate dehydrogenase. The assay mixture (1 ml) contained 50 mM Tricine (pH 8), 0.25 mM NADH, 5 mM DTT, 10 mM $MgCl_2$, 20 mM $NaHCO_3$, 0.02 U MDH (Sigma) plus enzyme extract. After 5 min incubation at 30°C, reaction was initiated by 5 mM PEP [12]. NADP-ME was assayed as previously described [1]. The results are the means of three measurements.

Immunological quantification. For immunochemical analysis, proteins were precipitated between 10 and 60% saturation of $(NH_4)_2SO_4$ and recovered by centrifugation at 48 000 *g* for 20 min. The resulting pellet was resuspended in a minimum vol. of 50 mM Tricine buffer, pH 8. For RuBPc, crude protein extract was used for immunological quantification. Rocket immunoelectrophoresis according to Laurell [13] was performed in 25 mM Tris buffer, pH 8, by addition of 186 mM glycine, containing either antiserum against Sorghum PEPc [7] or against Sorghum NADP-EM (Jolivet and Vidal, personal communication) or against Tobacco RuBPc (Prioul, personal communication). Each antiserum was added in liquid agarose at 50° (5 μl per ml agarose). After electrophoresis (50 V for 9 hr at 4°), the gels were dried, washed with a soln of 0.8% NaCl in water, then stained with Coomassie Blue soln for protein peak visualization. Rocket heights were measured and expressed in mm/mg of protein (arbitrary unit). The validity of the technique was tested for a definite range of soluble protein dilutions; proportionality between the hight of the rocket and the amount of soluble protein applied to the wells was established. All the subsequent measurements were restricted to this range (Fig. 2). The amount of enzyme protein in treated seedlings was based on the height of the rockets. It was calculated as the percentage of the height of the control rocket. Data above 100 mean an increase in the amount of protein in the presence of NaCl while numbers below 100 mean a decrease in the amount of protein as compared to the control.

Determination of soluble protein. Soluble proteins were assayed in extracts by using the protein dye binding procedure as described by Sedmak and Grossberg [14].

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