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Phosphoenolpyruvate Carboxylase Activity of Wheat and Maize Seedlings Subjected to Salt Stress

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Abstract: Phosphoenolpyruvate carboxylase (EC4.1.1.31) was extracted from maize (*Zea mays*) and wheat (*Triticum aestivum*) leaves. Measurements were taken one week after imposing the stress. PEPCase was assayed under 0 to 90mM NaCl. Inhibition of phosphoenolpyruvate carboxylase by NaCl depended on the source of the enzyme as well as on the concentration of the substrate (PEP). PEPCase isolated from leaves of C4 plants (maize) was extremely sensitive to the inorganic salts, whereas the enzyme extracted from C3 plants (wheat) was less sensitive. Our results also showed that the degree of inhibition of PEPCase activity is dependent on the concentration of PEP. About 33% inhibition of PEPCase was obtained at saturating level (2.2mM PEP) and 50% at sub saturating level (0.5mM PEP).

Keywords: Phosphoenolpyruvate carboxylase, Alloenzymes and Osmolality

INTRODUCTION

The activity of many enzymes is affected by addition of salts. Some enzymes show marked inhibition by inorganic ion concentration above 100mM (Greenway and Osmond, 1972). Concentrations higher than 100mM are frequently encountered in plant tissue during growth in saline environments. It has been proposed that salt – tolerant species are better in regulating the cytoplasmic ionic status and thus avoid interactions between enzyme and salt, which may be responsible for salinity damage in other species. Phosphoenolpyruvate carboxylase (EC4.1.1.31) is an important enzymic protein in higher plants functioning in malate synthesis. As a key enzyme in CAM, C4 photosynthesis and guard cell function, PEP carboxylase has been the subject of extensive experimental research in the last decades (Blanke *et al.,* 1986; Notton and Blanke, 1992 and Ting and Osmond, 1973). In C3 plants also, PEPCase plays an important role in providing oxaloacetate for the TCA cycle (Latzko and Kelly, 1983 and Melzer and Leary, 1987). Because of the distinctly different roles of malate in the leaves of C4 and C3 plants, it is possible that the kinetic and physical properties of the alloenzymes of PEP carboxylase in C4 and C3 species may differ. In this context, it is well known that the total activity of the PEPCase in C4 plants is greater than in C3 plants (Hatch and Slake, 1970; Jeanneau *et al.*, 2002).

In this work, we provide evidence of clear differences between properties of the PEPCase alloenzymes of maize (C4) and wheat (C3) species in response to salinity.

MATERIALS AND METHODS

Wheat grains (*Triticum aestivum*) and maize (*Zea mays*) were washed twice with dist. water, followed by soaking in 0.01M $HgCl_2$ for 2 min. then washing in dist. Water several times. Five–day old seedlings were grown hydroponically in growing units; each consists of 5 tubes of diameter 5cm and length 70cm. About 25 holes were made in each of the plastic tubes, and the seedlings were put each in a small eppendorf (after cutting about 4mm of the tip). The eppendorfs were then fixed in the holes.

Seedlings were irrigated with a complete strength Hoagland solution. An air pump (200ml/min.) was put in the inlet of the tube to provide good circulation and aeration of the solution. The pH was kept within the range of 6.0 to 6.5. The experimental design consisted of 4 different salinity levels made by the addition of 0.0 (control), 30, 60 and 90mM NaCl the nutrient solution. The solutions were made using dist. Water, and the volume of the solutions was maintained by adding dist. Water. The growth units were kept in a controlled chamber with temperature (28˚C), under a 13 hr. light / 11hr. dark cycle and a regime of light intensity of 200 $W.m^{-2}$ at plant height.

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Extraction of PEP Carboxylase:

Extracts were prepared 7 days after transferring the seedlings to the growth units. Approximately 1.0g of leaf tissue was ground in a prefilled mortar with purified sand and 10 ml of homogenization buffer, which has the following composition: 0.1M Tris HCl (pH 7.8), 0.5mM EDTA, 1mM $MgSO_4$ and 1mM DTE (freshly prepared).

The method is slightly modified from that described by Foyer *et al.* (1994) to suit the quantities of the enzyme present in the leaves investigated .The extract was centrifuged in a Beckman-centrifuge (model 2-21) for 25 min. at 16.000 rpm. Approximately 0.3g of solid PVP was added to 3ml of the supernatant and mixed vigorously with a stirrer for 3 sec., and then the mixture was centrifuged at 4°C for 10 min. at 8000 rpm. The clear supernatant was used as the source of the enzyme.

Assay of PEP Case:

Activity of PEPCase was determined spectrophotometrically as described by Blanke *et al.* (1986) at 340nm by coupling the reaction to the oxidation of NADH in the presence of malate dehydrogenase (MDH). The standard assay medium contained the enzyme extract, 10 units of MDH, 0.1mM NADH, 2.5mM $MgSO_4$ and 5mM $NaHCO_3$ in a total volume of 2.95ml 50mM Tricine buffer (pH 8.8). The reaction was started by the addition of 50 ml of PEP at 2.2mM final concentration. The rate of oxidation of NADH was measured 15 sec. after the addition of PEP over 3 min. The reaction was observed using the visual display of the spectrophotometer (Cecil CE 7200 split-beam spectrophotometer) to confirm the adequate mixing of the cuvette contents and that NADH oxidation caused by the reaction was linear. Assays were done in duplicate.

Cell-sap Osmolality:

Osmolality of the cell sap was measured using Osmomate (S^{TM} Model 2004) which was first calibrated with water and NaCl standard solution (1500m Osmol/Kg). The shoot juice was obtained by pressing 0.5g of shoots in pressing machine and using 1ml eppendorf vessel before being centrifuged for 1 min. at 6000 rpm using a cooling centrifuge (model ALC–Refrigerated–centrifuge PK 130R). The crystallization process of the supernatant in the osmomate vessel is automatically initiated and the crystallization temperature can be read at the digital display as the measuring value for osmolal concentration.

Statistical Analysis:

The experiment was a complete randomized design consisting of three salinity treatments, two species and three replications. Analysis of variance (ANOVA) was performed with the statistical program Minitab, involving two levels of classification (salinity and species) with interactions. Means were separated using the least significant difference (LSD) test at 5% level. LSD (0.05) values were compounded from ANOVA combinations as opposed to paired comparisons. For statistical comparisons the LSD (0.5) value can be used to compare the difference between any combination of two means within table or figure. The species by salinity level interactions were significant at $p < 0.05$ for all the ANOVA analysis where LSD (0.05) value has been presented.

RESULTS AND DISCUSSION

Increasing NaCl concentration in the growth medium significantly ($p < 0.1$) decreased PEPCase activity in both species. The activity of Phosphoenolpyruvate carboxylase in leaf extracts of maize and wheat seedlings subjected to different NaCl concentrations is represented in Fig. (1). Extracts of *Zea mays* leaves (C4 plant) showed much greater NaCl inhibition of the enzyme than extracts from *Triticum aestivum* (C3 plant). NaCl at a concentration of 90mM decreased maize PEPCase activity by 50% at sub saturating (0.5mM) PEP concentration, while the percent decrease in enzyme activity was 32% in wheat leaves. Fig. (2) shows the activity of PEPCase in leaves of maize and wheat assayed at saturating (2.2mM) PEP concentration. The maize enzyme activity was inhibited 21%, 27% and 33% by 30, 60 and 90mM NaCl respectively. The degree inhibition of the wheat enzyme, however, did not exceed 17% in plants subjected to 90mM NaCl. Fig. (3) shows that the cell sap osmolality of shoots increased with increase in salinity concentration (in both species). The increase in osmolality was higher in shoots of wheat than maize. Another effect of high salt concentration on many enzymes is to displace the pH optimum, leading to an apparent stimulation or inhibition of activities under standard assay conditions. Figs. (4 and 5) show that the pH shift, however, cannot account for the lowered activity of PEPCase shown in Fig. (1). Over a wide pH rang, NaCl dramatically inhibits the enzyme isolated from leaves of maize (C4 plant) but causes only small shifts in the pH optimum. Thus PEP carboxylase from leaves of C4 plants is very much more sensitive than the enzyme from leaves of C3 plants, confirming the data presented in Fig. (1).

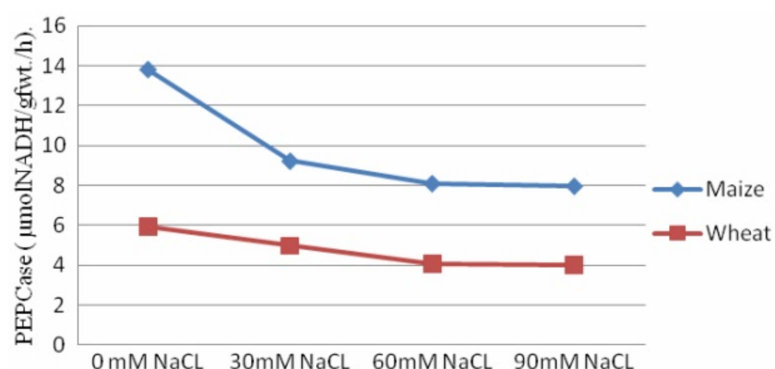

Fig. 1: Effect of different NaCl concentrations on PEP carboxylase extracted from leaves of maize and wheat after one week of exposure. Assay of PEPCase was done under sub-saturating (0.5mM) PEP concentration.

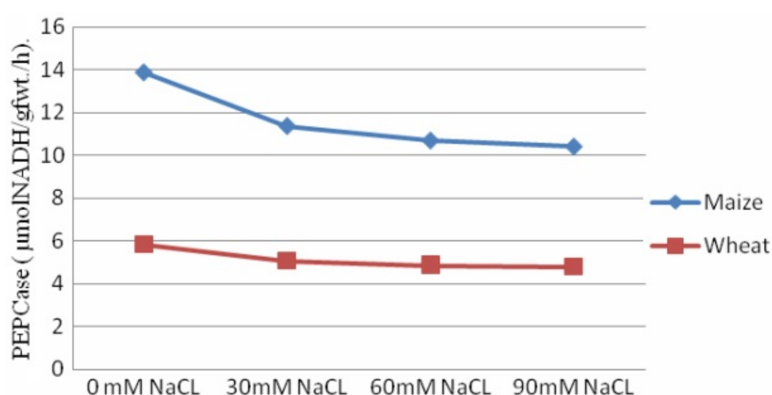

Fig. 2: Effect of different NaCl concentrations on PEP carboxylase extracted from leaves of maize and wheat after one week of exposure. Assay of PEPCase was done under saturating (2.2mM) PEP concentration.

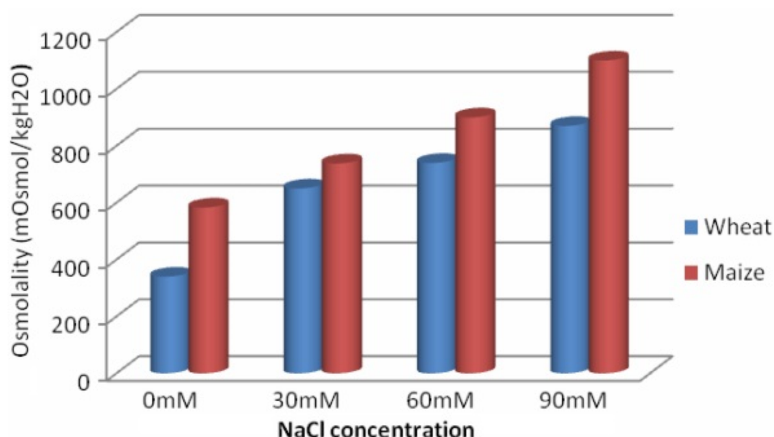

Fig. 3: Osmolality of maize and wheat shoots after one week of exposure to different NaCl concentrations in the nutrient medium.

C4 plants display a concentric organization of photosynthetic leaf tissues (mesophyll and bundle sheath) in which enzymes of the photosynthetic pathway, C4 cycle and Benson-Calvin cycle, are distributed (Mendez *et al*., 2000). In the C3 plant leaf, PEP carboxylase is no longer involved in photosynthesis, but fulfils a variety of physiological roles. In the anaplerotic pathway, that also occurs in C4 plants, the enzyme contributes to the replenishment of Krebs cycle intermediates when organic acids are directed towards other metabolic pathways

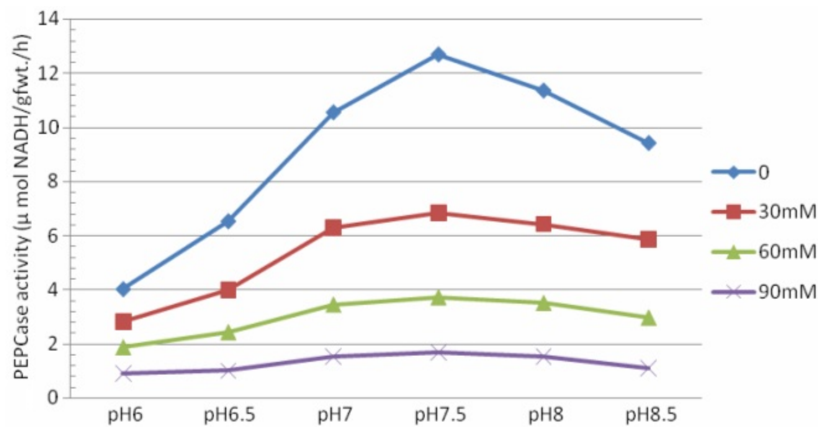

Fig. 4: The pH response for PEP carboxylase extracted from maize Seedlings subjected for one week to different NaCl concentrations.

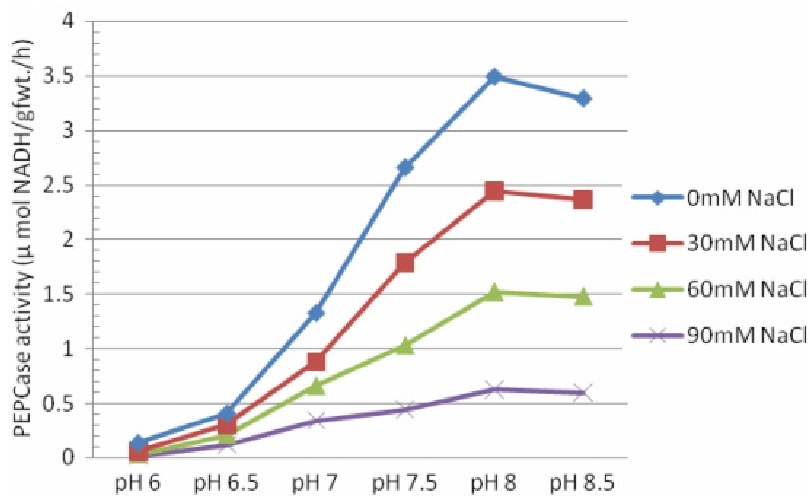

Fig. 5: The pH response for PEP carboxylase extracted from wheat Seedlings subjected for one week to different NaCl concentrations.

such as amino acid or protein synthesis (Stitt, 1999). The phosphoenolpyruvate carboxylase isolated from different species appears to vary in physical properties, as indicated by pH optima and response to NaCl. A clear example of this difference is the much higher salt sensitivity of PEP carboxylase isolated from leaves of C4 plants, compared with the same enzyme isolated from leaves of C3 plants. Our results also showed that, the degree of PEP carboxylase inhibition is dependent on the concentration of the substrate PEP e.g., PEPCase activity was inhibited 50% by 90mM NaCl when PEP was at a sub saturating concentration of 0.5mM.

The obtained results agree with those recorded by Notton and Blanke (1992) who isolated the PEPCase from "Fuerte" Avocado fruit, they concluded that chloride (180mM) caused an increase in apparent Km (PEP) from 80 to 212 accompanied by a 14% decrease in Vmax of the enzyme. Salt (NaCl) imposes several kinds of stresses upon plants. It causes drastic changes in the osmotic water balance and increase the cellular concentration of deleterious ions, as indicated by the significant increase in Osmolality of the cell sap. This may lead to membrane disorganization, ion toxicity and oxidative stress (Fouer *et al*., 1994 and Jeanneau *et at*., 2002). The PEPCase of mesophyll cells in C4 plants is loosely associated with the chloroplast (Dubey and Singh, 1999 and Slack, 1969).

It may be located in the peripheral reticulum, an elaboration of the inner membrane of the chloroplast envelope, or it may be a cytoplasmic enzyme. This carboxylase may thus be exposed to the lower cytoplasmic salt levels and exhibits correspondingly greater sensitivity to salt than chloroplastic enzymes.

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