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Relationship between ATPase activity and phosphoenolyruvate carboxylase (PEPC) capacity of durum wheat (*Triticum durum*) roots in conditions of salt stress

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Key words: Durum wheat, Salt stress PEPC capacity, Membrane ATPase activity.

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Abstract

The effect of NaCl on the membrane ATPase activity was studied at the root level in durum wheat (Triticum durum Desf. Var Karim). The salt treatment causes a stimulation of the ATPase activity and an acidification of the culture media. The high correlation between the PEPC capacity and ATPase activity shows that these two parameters are related. This statement was confirmed by the use of a specific proton pump inhibitors (ortho vanadate phosphor of Na+). The application of this proton inhibitors on plants limits the increase in the PEPC capacity which would be caused by an increase of cytoplasmic pH due to the membrane ATPase activity.

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Introduction

The problem of salinity is growing more and more particularly in the arid and semi arid and still a major obstacle of growth and development of plants (Davenport *et al.,* 2005; Hamrouni *et al.,* 2010). To perform against this constraint, the cytoplasmic osmotic adjustment is assured by the accumulation of inorganic ions and / or organic solutes such as proline, glycine betaine and sugars (Hu *et al.,* 2000; Sairam and Tyagi, 2004; Driouich, 2006). These metabolites also called "osmoprotectants" could ensure the protection of plant cellular organelles against the toxic effects of salts especially the ions Na^+ and Cl^-. They can protect the protein structures and ensure some biochemical reactions (Tozlu *et al.,* 2000) which some of them were used by several authors for the improvement of the tolerance of plants to salinity (Grumet and Hanson, 1986; Rhodes *et al.,* 1989).

On the other hand, the accumulation of the production of oxaloacetic acid and other organic acids which derive from them, including the malate, could play an important role in regulation of cytoplasmic pH (Fukada-Tanaka *et al.,* 2000) and in maintenance of the cellular electroneutrality face of the excess of cations absorbed, particularly Na^+ (Latzko and Kelly, 1983). The increase of the cytoplasmic pH in conditions of salt stress could stimulate the ability of the PEPC (Guern *et al.,* 1983) induce by the change in activity of proton pump inhibitors (Romani *et al.,* 1983). Some authors have shown that the NaCl generates an activation of the ATPases and Na^+/H^+ antiporter of the plasma membrane (Niu *et al.,* 1993).

In this work we studied the evolution of the membrane ATPase activity of durum wheat (*Triticum durum*) roots in the conditions of salt stress in order to determine the relationship between the capacity of PEPC and the ATPase activity.

Materials and methods

Plant material

The "Karim" variety of durum wheat (*Triticum durum* Desf.) was chosen for its tolerance to NaCl at the stage after germination (Driouich, 2006). The choice of roots for studying action mode of NaCl is justified that they are the first bodies of plant which are in direct contact with salt environment.

Experimental protocol

The test was conducted in hydroponic culture. The 20 seeds selected were disinfected with sodium hypochlorite (1%), washed in distilled water and geminated petri dishes (containing the filter paper dampened with distilled water0 in the dark at 20°C. After emergence of the radicle plant in three following days, the seedlings were transplanted in crystallizers (0,2 m^3 of diameter) containing Hoagland nutrient solutions at 6.3 pH (Hoagland and Arnon, 1938). Different concentrations of NaCl (0,50, 100 and 150 mm) were added to the culture media. To avoid significant variations in pH caused by the ionic imbalance, the nutrient solutions were continuously aerated and renewed every two days. The cultures were placed in a growth chamber with following conditions: lighting of 300 mmol/m2/s2 PAR, 16 hours of photoperiod, temperature of 18°C night and 24°C day, a humidity of 60% day and 80 % night.

The experimental scheme adopted is a block five repetitions of 20 seedlings by crystallizer for each treatment. The tests was done on the seedling roots of 21 days old.

Methods of analysis

Measurement of PEPC capacity and malate content

Preparation of "crude extract"

The "crude extract " are prepared according to the Warrior method (1988). For each crystallizer, the roots are separated from the aerial parts of plant and after rinsing them in distilled water. The dry matter of leaves and roots is determined by drying them in oven at 80°C for 48 hours. 500 mg of dry matter is collected, crushed with 2 ml of buffer Tris-bicine 100 mM pH 8. The homogenate is centrifuged at 10,000 g for 10 minutes and the supernatant obtained represents "crude extract". All extraction steps were

performed at 4°C as soon as possible to minimize the process of proteolysis.

Measurement of PEPC capacity

The PEPC capacity is measured in vitro in the crude extract. It represents the activity of the enzyme in optimum conditions of substrates and cofactors (Queiroz and Morel, 1974). We follow the oxidation of NADH for at least 10 min at 30°C in buffer solution of ris-bicine pH 8. The reading is performed by spectrophotometer (Beckman DU 640) at 340 nm

Determination of malate content

The malate is assayed in the "crude extract" according to the method of Hohorst (1970) in the presence of malate deshydrgenase enzyme. The measurement is carried out in a buffer solution of hydrazine- glycine (400 - 500 mM) pH 9, in 2.5 mm of NAD and 40 µl of the "crude extract". A first reading of the optical density (OD_1) is made with a spectrophotometer at 340 nm. The second reading (DO_2) is obtained after 10 minutes of adding 5 UE of trade MDH (Sigma).

Measurement of the ATPase activity

The roots are washed in cold distilled water, dried and then put in plasmolyse for 40 minutes in a homogenization buffer solution of Tris-HCl 100 mM pH 7.5 containing 5 mM EDTA, 10 mm beta-mercaptoethanol, 0.5 M sucrose and 40 µM PMSF. Then they are crushed cold and the homogenate is filtered through layers gauze. The filtrate obtained underwent a series of centrifugation until the obtaining of "enzyme extract" (Driouich , 2006).

All extraction steps were also performed at 4°C. The membrane ATPase activity is measured according to the method of Chen et al. (1956) on "extract enzyme" in a mixture reaction containing 3 mM ATP, 3 mM Mg SO_4, 50 mM KCl, 0.02 % Triton X-100, 2 mM sodium molybdate and 1 mM Na Azide. The reaction was held at 38°C for 15 min; it is initiated by adding the enzyme extract and stopped at 0°C by 2 ml of reagent (ammonium heptamolybdate 0.5% in H_2SO_4 1N and ascorbic acid 2% in H_2SO_4 1N).

The volume of the reaction media is brought to 500 µl by the Tris-Mes buffer solution of 50 mM, pH 6.5. The blue coloration is developed during 40 min at ambient temperature. The tubes are reset to 0°C to stabilize the coloration and optical density is measured by spectrophotometer at 820 nm.

Result

The evolution of the membrane ATPase activity of roots in durum wheat variety "Karim" is represented on the figure 1. It shows that the salt treatment of *seedling's roots* for 4 days led to ATPase activity stimulation and an acidification of culture media which could be explained by the release of H^+ protons.

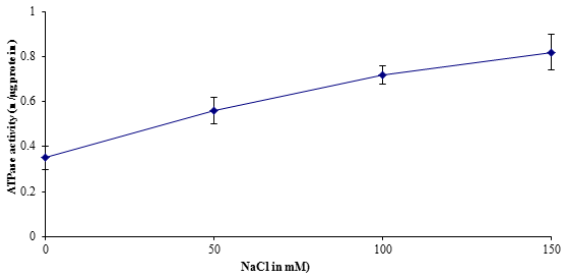

Fig 1. Variation in the membrane ATPase activity of wheat roots under salt treatment for a period of 4 days

Transfer of seedlings under normal conditions for 48 hours resulted in a return of ATPase activity to standard level (Table1). The pH of the culture media has not changed. These results show that the membrane ATPase activity is reversible.

Table 1. Evolution of the ATPase activity after transfer of seedlings in normal conditions during 48h

NaCl (In mM)	*ATPase Activity (In µmol. $^{-3}$.min^{-1}.µg^{-1} 1 protein)*
0	0.40 +- 0.12
50	0.42 +-0.10
100	0.46 +-0.08
150	050 +- 0.15

On the other hand, the high correlation (r = 097 ***, p < 0.1 %) between the PEPC capacity and membrane ATPase activity of wheat roots under salt stress suggests that the increase of PEPC capacity and malate content (Figures 2 and 3) could be related to the increase of proton pump activity caused by NaCl.

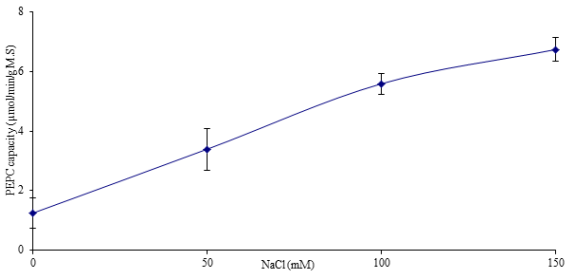

Fig. 2. Variation in PEPC capacity of wheat roots under salt stress for a period of 4 days

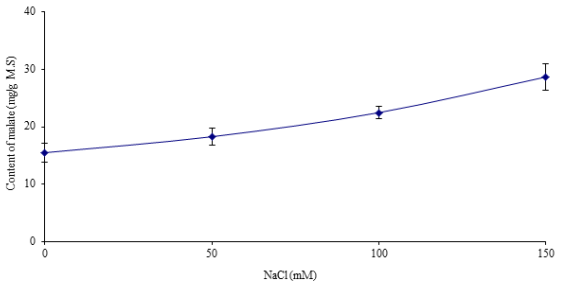

Fig. 3. Variation in malate content of wheat root under salt stress conditions for a period of 4 days

To verify this hypothesis, we used a specific inhibitor of the proton pump (ortho vanadate Na^+) applied for 4 days at concentration of 300 µM in the same time as NaCl. The results obtained shows that inhibition of the proton pump by the orthovanadate Na^+ limits the increase of the capacity of PEPC (Figure 4) and the content of malate of roots caused by NaCl (Figure 5).

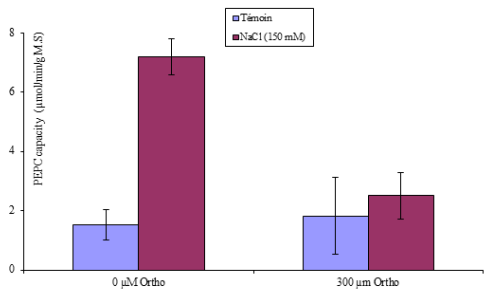

Fig. 4. Effect of 4 days treatment with Na orthovanadate (300 microM) on the PEPC capacity of wheat roots

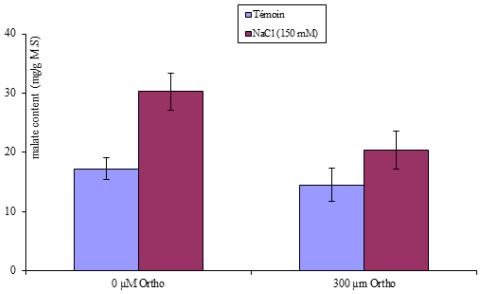

Fig. 5. Effect of 4 days treatment with Na orthovanadate (300 microM) on the malate content of wheat roots

On the other hand, we have checked the action of IAA (50 µM) as an ATPase stimulator in the absence or presence of Na^+ ortho vanadate (300 µM). The results obtained (Figures 6 and 7) show that the AIA alone causes an acidification of the external environment and an increase of the PEPC activity and the malate content. This acidification is deleted by proton pump activity (Na^+ ortho vanadate phosphor) which also increase the stimulation of the PEPC capacity and the malate content observed in the presence of the AIA.

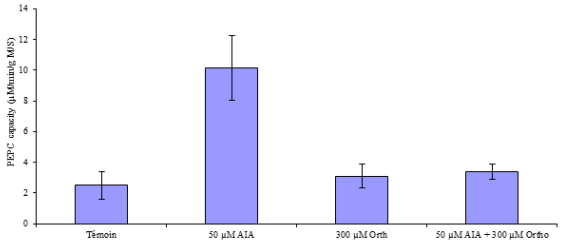

Fig. 6. Effect of 4 days treatment by 3-indolyl acetic acid (AIA: 50 microM) acid in the presence or absence of Na+ orthovanadate (300µM) on the PEPC activity of wheat roots.

Discussion and conclusion

The effect of NaCl on the membrane ATPase activity was studied at the level of the durum wheat roots variety "Karim". We have shown that the NaCl stimulates the membrane ATPase activity in the roots and increases the PEPC capacity and the malate

content. The synthesis of malate in response to salt stress could play the osmoticum role and contribute in adaptation mechanism to salt stress. The important PEPC activity in the presence of NaCl can be interpreted as a process of regulating the excessive inorganic cations penetration by organic acids mainly the malic acid. The same results were obtained in several species of wheat by Latzko and Kelly (1983), Whittingten and Smith (1992), Osmond and Popp (1983).

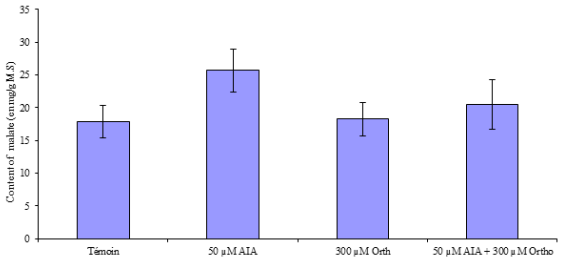

Fig. 7. Effect of 4 days treatment by 3-indolyl acetic acid (AIA: 50 microM) acid in the presence or absence of Na+ orthovanadate (300µM) on the malate content of wheat roots

The high correlation between PEPC capacity and ATPase activity shows that these two parameters are closely related. Indeed, it is likely that alkalinization of cell media following the output of H^+ is responsible of the increase in PEPC activity. As result, the production of acid oxaloacetique and other organic acids which derive from them, including the malate, could play a crucial role in the regulation of the cytoplasmic pH (Fukada-Tanaka *et al.,* 2000) and in the maintenance of the cell electroneutrality, in response of cations excess absorbed by Na^+ (Latzko and Kelly, 1983). The role of ATPase has been clarified by the use of a specific inhibitor (Orthovanadate of sodium) which limits the increase of PEPC capacity and malate content in wheat roots caused by the salt treatment. Therefore, the increase of PEPC activity would be due to the alkalinization of the cytoplasm subsequent the activation under effect of salinity of the proton pump. For that reason, the stimulation of the PEPC may be caused by an increase of the cytoplasmic pH (Guern *et al.,* 1983) due to changes in activity of proton pumps (Romani et al. , 1983). Other authors have shown that the NaCl induces an activation of the Na^+/H^+ antiporter of the plasma membrane (Kartz *et al.,* 1992; Niu *et al.,* 1993; Tracey *et al.,* 2009; Yuda, 2011).

The role of PEPC in maintenance of intracellular pH is confirmed by the use of indolacetic acid. Its application in low concentrations causes the efflux of H^+ leading to a cytosol alkalization and an increase of PEPC capacity and malate content in the wheat roots. Therefore, it appears that the increase in the PEPC activity would be caused by an elevation of cytoplasmic pH due to the activation of the membrane ATPase.

In conclusion, the PEPC activity observed in the variety "Karim" in under salt stress, coupled with the pumps protons, appears crucial to avoid the change in pH of roots cells cytoplasm caused by the excessive accumulation of Na^+ and Cl^- (Driouich *et al.,* 2001). On the other hand, synthesis of malate could play a osmoregulator role in condition of salt stress.

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