

EFFECT OF SALINE STRESS ON THE ACTIVITY OF *VIGNA UNGUICULATA*H<sup>+</sup>-PYROPHOSPHATASEEFEITO DO ESTRESSE SALINO SOBRE A ATIVIDADE DE H<sup>+</sup>-PIROFOSFATASE DE *VIGNA UNGUICULATA*

Maria Clara Guimarães<sup>1\*</sup>, Carlos Eduardo de Souza Teodoro<sup>1\*</sup>,

<sup>1</sup> Universidade Federal Fluminense

\* Autor para correspondência: [eduardo@metal.eimvr.uff.br](mailto:eduardo@metal.eimvr.uff.br)

**ABSTRACT**

The effect of saline stress on H<sup>+</sup>-PPase activity of membranes from different organelles from the secretory pathway of *Vigna unguiculata*, was evaluated by isolating hypocotyl 's membrane vesicles from plants treated with NaCl for 48 hours. The exposure of seedlings to NaCl 200 mM, stimulated the transport activity of H<sup>+</sup> by H<sup>+</sup>-PPase in total membranes of *V. unguiculata*. When the hydrolytic activity of the present enzyme in different organelles from the secretory pathway were analyzed, it was noticed that exposure to saline stress reduced the activity of this enzyme, located in the tonoplast, in addition, it stimulated the activity of the present enzymes in Golgi's apparatus, endoplasmic reticulum + plasmatic membrane (ER+PM) and nuclear envelope (NE). The highest stimulus of PPI's hydrolysis activity by H<sup>+</sup>-PPase was observed in the Golgi's membranes. These results indicates that these organelles may be equipped with different enzymes' isoforms, that responds differently to salt stress.

**Key words:** secretory pathway. Epace10. Cowpea. NaCl.

## RESUMO

O efeito do estresse salino sobre a atividade de  $H^+$ -PPase de membranas de diferentes organelas da via secretória de *Vigna unguiculata* foi avaliado, isolando-se vesículas de membranas de hipocótilos de plantas tratadas com NaCl durante 48 horas. A exposição das plântulas a NaCl 200mM estimulou a atividade de transporte de  $H^+$  por  $H^+$ -PPase em membranas totais de *V. unguiculata*. Quando analisada a atividade hidrolítica da enzima presente em diferentes organelas da via secretória, observamos que a exposição ao estresse salino reduziu a atividade da enzima localizada no tonoplasto e estimulou a atividade das enzimas presentes no Golgi, Retículo endoplasmático + membrana plasmática e envelope nuclear. O maior estímulo da atividade de hidrólise de PPi por  $H^+$ -PPase foi observado nas membranas do Golgi. Estes resultados indicam que estas organelas podem estar equipadas com diferentes isoformas da enzimas, que respondem de maneira diferente ao estresse salino.

**Palavras-chaves:** Via secretória. Epape 10. Feijão de corda. NaCl

The  $K^+$ -stimulated vacuolar PPase, is one of the major components of the plant cell's tonoplast. In some cases, when combined with  $H^+$ -TIP and V-ATPase, it can generate a proton gradient of similar magnitude to the one generated by  $H^+$ -ATPase in the tonoplast of some species (1). However, differently from V-ATPase, the V-PPase obtains its energy from pyrophosphate hydrolysis (PPi) (2, 3).

V-PPases were cloned from different organisms like terrestrial plants, green and marine algae, photosynthetic bacteria, protozoa and archaeobacteria (4). The first cDNA for  $H^+$ -PPase clone was the *Arabidopsis thaliana*, exhibiting an AVP1 isoform (5)  $K^+$ -dependent, and more recently the cDNA was characterized to a  $K^+$ -independent isoform, the AVP2 (6). For *Beta vulgaris* (7) two isoforms with mass of 80550 and 80000 Da were isolated showing an identity of 88 and 89% with  $H^+$ -PPase of *A. thaliana*, respectively.

Several roles have been suggested for V H<sup>+</sup>-PPase in plant cells. The enzyme can work like a power supply system by a gradient of pH through the tonoplast, which is used to energize the secondary active transport. And next to V H<sup>+</sup>-ATPase, it may also contribute to the regulation of cytosolic pH (8, 9). It has been suggested that in the coleoptiles of corn seeds, the gradient of H<sup>+</sup> generated by V-PPase and V-ATPase can be used by the H<sup>+</sup>-PPase to synthesize the ATP e PPi, respectively, showing a reversibility character of these H<sup>+</sup> pump (10). Under conditions where the ATP supply is limited, H<sup>+</sup>-PPase would have the role of generating and maintaining the H<sup>+</sup> electrochemical gradient through the tonoplast (11).

The existence of different V-PPase isoforms in the plant genome suggests a differential regulation of this enzyme during the developmental stage by the tissue's specificity or the regulation of the enzyme's expression by various environmental factors. Stress caused by the soil's high salinity is one of the main challenges for non-halophilic plants. It imposes serious constraints on agriculture in many areas around the world, particularly, in irrigated agriculture (12). Saline soils are one of the main limiting factors in the world's agriculture. Around 20% of all arable land in the world and approximately 50% of irrigated land are affected by salinity (13). Moreover, salinization due to irrigation has increased greatly in the world (14).

Several studies have been focused on the regulation of H<sup>+</sup>-PPase activity by the growth of plants in the presence of salt. In contrast to what is observed for V-ATPase activity, where NaCl generally induces an increase of the enzyme's activity, the tonoplast's V-PPase activity shows a variable response for the plant's exposure to NaCl (15, 16, 17). In Suaeda parsley, a native plant of saline soils in China, the activity of V-PPase K<sup>+</sup>-stimulated, varied according to the saline treatment that was given to it. When the plants were treated with 0.1 M NaCl, there was a linear increase in V-PPase activity. In the treatment with 0.4 M NaCl, the highest V-PPase activity was observed after two days, and on the 16<sup>o</sup> day, the pyrophosphatase activity was significantly lower when compared to the control one (16).

This work aimed to verify the effect of saline stress by the activity of H<sup>+</sup>-PPases located in the membranes of different compartments of the *Vigna unguiculata* secretory's pathway.

The cultivar used was selected for cultivation in the northeast region and is known to tolerate high temperature and water deficit conditions

Seeds of cowpea *V. unguiculata* Walp cv. EPACE 10 were used in this work. The cultivar is known to tolerating high temperature and water deficit conditions. The seeds of *V. unguiculata* were germinated on filter paper and grown during five days, at 25 °C ± 1 °C in the dark.

In order to verify the effects of saline stress on H<sup>+</sup>-PPase activity, *V. unguiculata* seedlings were treated for 48h before harvest with 200mM of NaCl.

Organelle membranes from secretory pathway of *V. unguiculata* were isolated as described by (18), from the drawn hypocotyls. From the total membranes obtained, one part (1.2-1.6 mL) was applied over a sucrose density gradient at concentrations of 20-56%. Pyrophosphatase activity was determined as described by (19). The hydrolytic activity of H<sup>+</sup>-PPase was determined by the concentration of inorganic phosphate released in the hydrolysis of the pyrophosphate by the enzyme. Determination of H<sup>+</sup> transport was performed as described by (20). Proton transport was measured by the decrease of the ACMA fluorescence when PPi was added to the assay buffer.

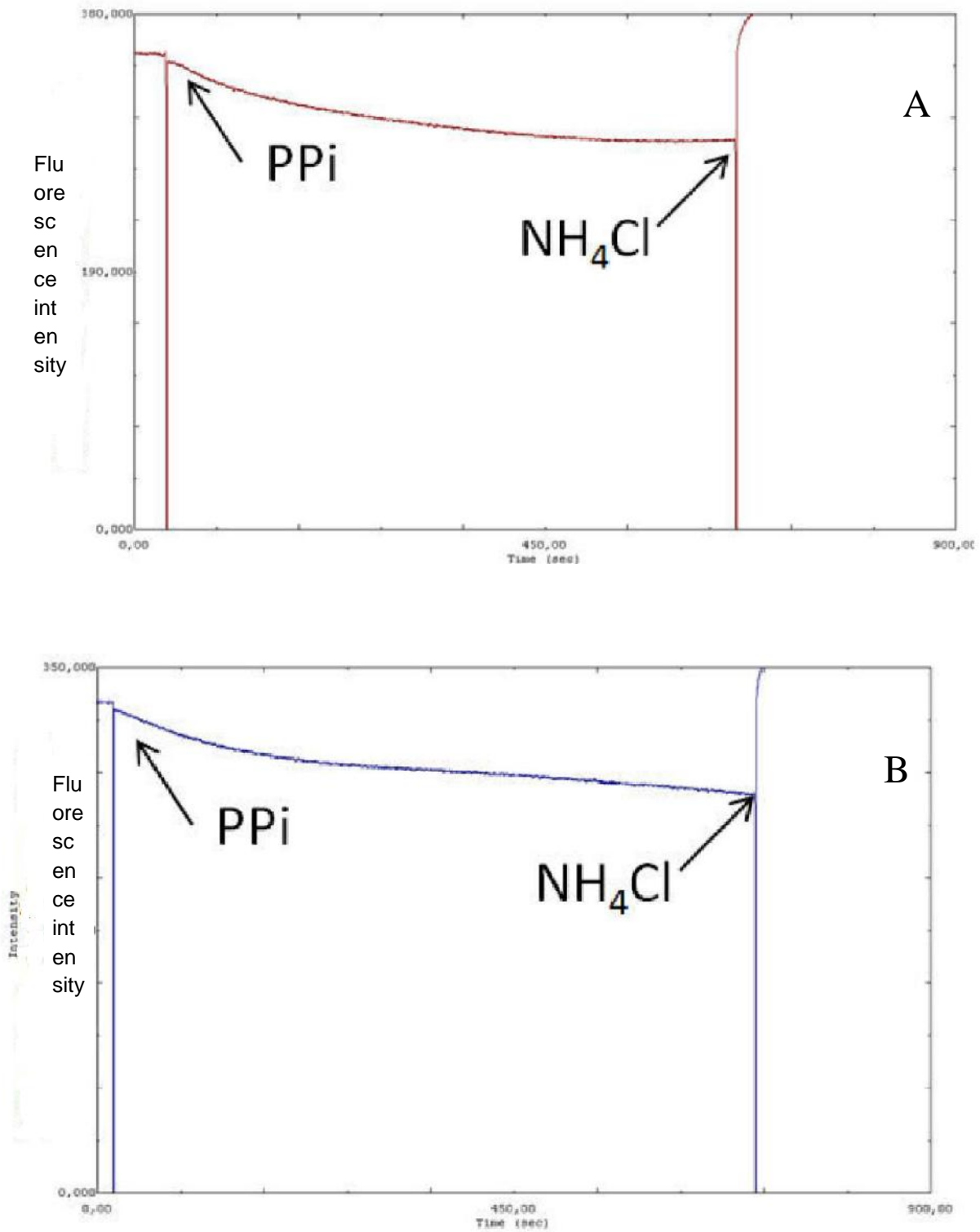
After the *V. unguiculata* seedlings were subjected to saline stress (200 mM NaCl) for 48 h, isolation of organelles membrane from the secretory pathway was performed. Formation of different bands separated in sucrose density gradient was observed (Figure 1). In this isolation, it was possible to separate the membranes of the vacuole, Golgi complex, membranes of the endoplasmic reticulum, plasma membrane and nuclear envelope.



**Figure 1.** Separation of membranes of organelles from the *V. unguiculata* secretory pathway in Sucrose density

In order to verify the effect of saline stress on the  $H^+$ -PPase activity of membranes from different organelles from secretory pathway of the *V. unguiculata*, isolation, fractionation and analysis of hypocotyl membranes from this plant were performed. As a result, it was possible to observe  $H^+$  transport dependent on PPi in total membranes of plants submitted to 200 mM NaCl as well as in the total membranes of those plants that were not submitted to saline stress (control). The transport of  $H^+$  can be visualized by the drop in fluorescence intensity.

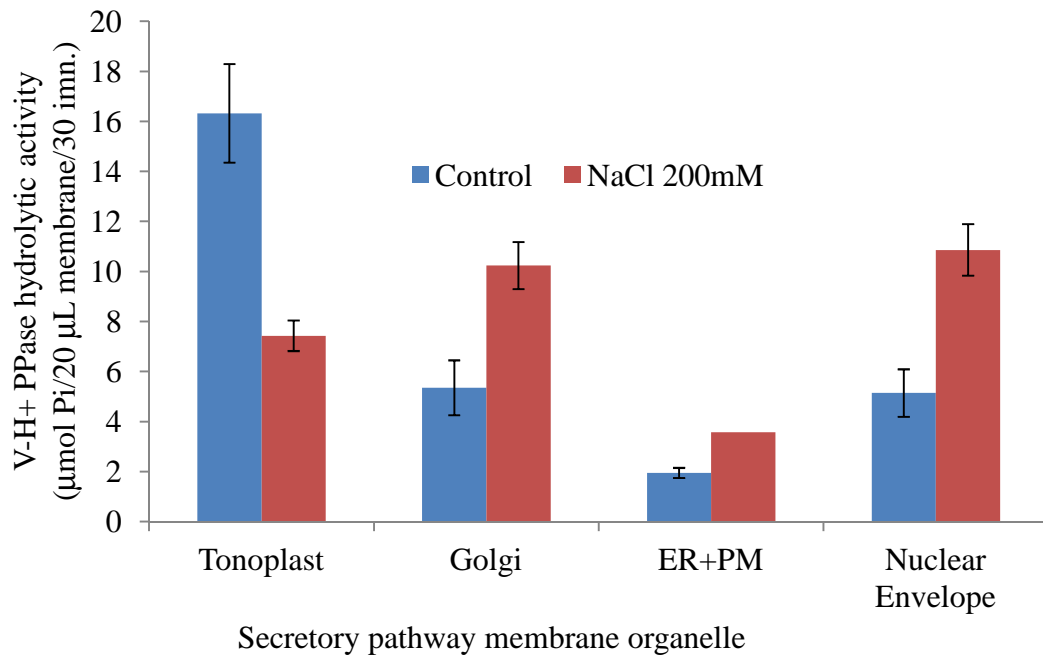
On figure 2 is observed that the exposure of seedlings to 200mM NaCl did not affect the transport of  $H^+$  dependent on PPi, in total isolated membranes from the styolated hypocotyl of *V. unguiculata*. It is important to emphasize that total membranes of seedlings organelles submitted to 200mM NaCl present lower protein content than those which were not submitted to saline stress (data not shown).



**Figure 2** Transport of H<sup>+</sup> by H<sup>+</sup>-PPase, in total membranes isolated from Etiolated hypocotyls of *V. unguiculata*. (A) control plants (B) Plants submitted to 200 mM NaCl. The proton transport was determined by measuring the fluorescence quenching of ACMA. The reactions were started by the addition of PPI 1 mM to the assay medium containing the membrane vesicles.

When the effect of saline stress on the activity of  $H^+$ -PPase is analyzed, It is possible to conclude that there was reduction on the activity of enzymes located in the tonoplast of plants which were submitted to 200 mM NaCl (Figure 3). On the other hand, for  $H^+$ -PPase located in Golgi's membrane, RE+MP and nuclear envelope, the NaCl exposure promoted an increase in the hydrolytic activity of  $H^+$ -PPase, in the order of 91.40%, 83.1% and 111.3%, respectively. These results indicate that enzymes located in those organelles react in different ways due to saline stress. Our results differ from those by (21), when was verified the culture of *Solanum tuberosum* cells exposed to 150mM of NaCl had the hydrolytic activity of  $H^+$ -PPase of tonoplast increased in 2-3 times. The same results were observed by (22), in which treatment with 200 mM of NaCl stimulated the activity of V- $H^+$ -PPase present in tonoplast of *Spinacia oleracea*. However, the results were not the same for *Salicornia dolichostachya*, a Halofite plant tolerant to high concentrations of sodium. This difference in the hydrolytic activity of  $H^+$ -PPase present in the different organelles of secretory pathway of *V. unguiculata* seems to demonstrate that they have different isoforms of this  $H^+$  pump and that they must have different roles regarding the tolerance of *V. unguiculata* to stress by  $Na^+$ .

Future researches may be developed to verify the effect of saline stress by the transport of  $H^+$  dependent of PPi in different organelles of the secretory pathway of *V. unguiculata*.



**Figure 3** Effect of saline stress on the hydrolytic activity of H<sup>+</sup>-PPase from different organelles of the *V. unguiculata* secretory pathway. The H<sup>+</sup>-PPase hydrolytic activities were measured as the liberation of the inorganic phosphate released from PPi. The amount of Pi released was colorimetrically determined as previously described. The results are means ± SD of three independent experiments.



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