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PROLINE LEVEL AND AMYLASE AND ASCORBATE PEROXIDASE ACTIVITY IN THE GERMINATION OF *Plantago ovata* FORSK (PLANTAGINACEAE) SEEDS

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ABSTRACT

The species *Plantago ovata* has great importance for the pharmaceutical industry due to the high mucilage level in its seeds. Thus, the present study aimed to verify proline level and amylase and ascorbate peroxidase activity in the germination of seeds subjected to stress, which was induced by using PEG, NaCl and $CaCl_2$ solutions. Fifty seeds were placed into filter paper moistened with 15 mL of such solutions at the potentials 0,-0.2, -0.4, -0.6 and -0.8 MPa in the presence of light at 25ºC. Proline levels decreased during *Plantago ovata* germination under water and salt stress, whereas the activity of ascorbate peroxidase and amylase was higher under PEG-induced stress, relative to those induced by NaCl and $CaCl_2$.

Keywords: proline level, amylase, ascorbate peroxidase, *Plantago ovata* seeds, water stress.

INTRODUCTION

Plants under environmental stress may present increased levels of certain compounds. According to literature, proline accumulates in leaves as a response to such stress types, especially water stress.

Several works have reported higher proline levels in plants subjected to water stress conditions. Hsu *et al.* (2003) studied the effect of PEG-induced water stress at -1.5 MPa and observed accumulation of proline and its precursor's glutamic acid, ornithine and arginine in rice leaves.

Baisak *et al.* (1994) studied the effect of PEG-induced water stress in wheat leaves at 1.5 MPa and observed a remarkable proline level increase. Lopes and Arrieta-Maza (1991) evaluated bean plants subjected to the same water potential and reported higher proline accumulation in the flowering stage than in the vegetative one; besides, the drought-tolerant cultivar presented higher proline accumulation than the susceptible one. Fumis *et al.* (1993) observed higher proline and total amino acid levels in leaves and roots of wheat seedlings cv. IAC-24 subjected to water deficit. Chen and Kao (1993) also detected proline accumulation in rice leaves subjected to water stress. According to Hanson (1980), proline accumulation in rice, sorghum, maize, wheat and barley started when the leaf water potential was reduced to -1.0 MPa.

Some authors have suggested the following functions regarding proline accumulation: stabilization of subcellular structures; energy storage as an osmotic adjustment mechanism and constitution of cell wall proteins. The high correlation between proline accumulation and drought-tolerance increase has also been described; however, such accumulation can be only a stress effect (Delauney and Verma, 1993; Madan *et al.*, 1995; Hare and Cress, 1997; Parida and Das, 2005).

Proline is an amino acid synthesized from glutamate or ornithine since both pathways contribute to proline synthesis under normal physiological conditions. However, the glutamate pathway predominates in cells under osmotic stress (Bartels and Nelson, 1994).

Reactive oxygen species are originated under water stress conditions. They oxidize photosynthetic pigments, membranes, lipids, proteins, and nucleic acids (Smirnoff, 1993; Alscher *et al.*, 1997; Yordanov *et al.*, 2000). Thus, antioxidants as carotenoids, ascorbate, α-tocopherol, glutathione and flavonoids, as well as antioxidant enzymes such as peroxidases, superoxide dismutase and catalase, can be synthesized in order to protect the plant cells (Tanaka *et al.*, 1990).

Several studies have been carried out to verify the action of some enzymes that indicate different types of environmental stress, especially water stress. Among such enzymes, amylase and ascorbate peroxidase may be included. According to literature, a higher activity of those enzymes has been detected under several stress situations in order to protect the plants against such adversities, favoring thus their survival (Koster, 1991; Hare and Cress, 1997; Nepomuceno, 2001; Taiz and Ziger, 2002).

Ascorbate peroxidase is an antioxidant enzyme that participates in the ascorbate-glutathione cycle and acts in chloroplasts and in the cytosol. It reduces H_2O_2 to H_2O by using ascorbate as reducer agent, protecting thus the plant (Asada, 1992; Meloni *et al.*, 2003).

The action of antioxidant systems under drought has been investigated by many authors in several crops, such as spinach (Tanaka *et al.*, 1990), pea (Moran et al., 1994), sorghum and sunflower (Zhang and Kirkham, 1996) and wheat (Sgherri *et al.*, 2000). Studies involving seeds during germination has not been frequent.

The aim of this study was to evaluate proline levels and amylase and ascorbate peroxidase activity in the

germination of *Plantago ovata* seeds subjected to water and salt stress.

MATERIAL AND METHOD

This work was carried out in the labs of the Department of Botany and Department of Chemistry and Biochemistry, Institute of Biosciences, São Paulo State University- UNESP-Botucatu Campus, São Paulo State, Brazil. *Plantago ovata* seeds were supplied by the Company "Centroflora Anidro"; they were from Canada, under the lot number 043214, and presented 98% germination.

Biochemical evaluations were performed to quantify proline levels and to determine the activity of amylase and ascorbate peroxidase. Experimental design was completely randomized, with 15 treatments and 4 replicates. Treatments consisted of a factorial arrangement with 5 potentials and 3 osmotic agents. Means were compared through Tukey's test at 5% significance. Percentage data were transformed into x+0.5 and the original values were presented.

Four replicates of 50 *P. ovata* seeds were placed into gerbox in filter paper moistened with 15 mL of polyethylene glycol 6000 (PEG), NaCl or $CaCl_2$ solutions at the potentials 0, -0.2, -0.4, -0.6 and -0.8 MPa, and were allowed to germinate at 25ºC in the presence of light. The Table cited by Villela *et al.* (1991) was used to correlate PEG concentration and osmotic potential. As regards NaCl and $CaCl_2$, the Van't Hoff equation mentioned by Salisbury and Ross (1992) was employed. Biochemical evaluations were performed after 48h. Seeds were considered germinated when they presented approximately 2mm root.

Proline quantification

Proline was quantified according to the method described by Bates *et al.* (1973). Seeds were taken from the gerbox after 48h, homogenized with 2mL ethanol 40% in a cold mortar containing a small amount of washed sterile sand. After agitation for 10 min, the extract was filtered through Whatman No. 2 filter paper and a 2mL-aliquot was used. Absorbance was measured in spectrophotometer at 528nm. Results were expressed as µmol proline per mg fresh matter and absorbance values were compared with proline standard curve.

Amylase assay

Seeds were taken from the gerbox and homogenized with 20mL sodium phosphate buffer 0.1 mol L^{-1} (4ºC) pH 7.5 in a cold mortar containing a small amount of washed sterile sand. After centrifugation at 10.000 x g (12.000 rpm) for 30 min at 4ºC, the supernatant was used as the enzymatic source, which was frozen at 4ºC until the amylase assay.

Amylase activity was determined by adapting the method of Caraway (1959). The reaction mixture consisted of 1mL enzymatic extract and 5mL sodium phosphate buffer / benzoic acid 0.5 mol L^{-1} pH 7 containing 0.1% (w v^{-1}) starch. After incubation for 30 min at 37ºC in water bath, 1mL iodine solution 0.01 mol L^{-1} was added, and the volume was completed to 10mL with distilled water. Absorbance readings were done in spectrophotometer at 660nm and compared with starch standard curve. The quantity of digested starch was calculated through the difference between the starch present in the reaction mixture and the remaining one. Amylase activity was expressed as mg digested starch per min per mg protein.

The quantity of protein in the enzymatic extract, which was used in the amylase assay, was measured according to the method of Lowry *et al.* (1951). Absorbance readings were done in spectrophotometer at 660 nm by using crystalline bovine serum albumin (BSA) as reference.

Ascorbate peroxidase assay

Ascorbate peroxidase was extracted according to the method described by Rama-Devi and Prasad (1998). Seeds were taken from the gerbox and homogenized with 5mL Tris-HCl buffer 50 mmol L^{-1} pH 7.8 in a cold mortar containing a small amount of washed sterile sand. After centrifugation at 10.000 x g (12.000 rpm) for 20 min at 4^0C, the supernatant was collected and used to assess ascorbate peroxidase activity according to the method of Nakano & Asada (1981). The reaction mixture consisted of enzymatic extract, 50 mmol L^{-1} sodium phosphate buffer (cold), pH 7, 0.5 mmol L^{-1} ascorbate, 0.1 mmol L^{-1} hydrogen peroxide and 0.1 mmol L^{-1} EDTA, in a 0.3mL final volume. The reaction started after the hydrogen peroxide addition, and the absorbance was measured in spectrophotometer at 40s interval at 290nm. The molar extinction coefficient 2.8 $mmol^{-1}$ cm^{-1} was used to calculate ascorbate peroxidase activity.

RESULTS AND DISCUSSIONS

The original data regarding proline level and amylase and ascorbate peroxidase activity, besides their respective variance analyses, are presented in the appendix (Tables 3 to 5).

The stress originated by using the three osmotic agents PEG, NaCl and $CaCl_2$ at the potentials -0.2, -0.4, -0.6, and -0.8 MPa significantly decreased proline levels relative to control (0.0 MPa) during *P. ovata* germination (Figure-1). Such result disagrees from those in literature, in which a gradual proline increase has been described in leaves as a response to stress situations, as observed by Sawasaki (1981) in bean plants, by Fumis *et al.* (1993) in leaves and roots of wheat seedlings, and by Hsu *et al.* (2003) in rice plants. However, those works used developed plants rather than seeds in germination, which suggests a differentiated behavior for the effect of water potential on proline levels in *P. ovata* seeds.

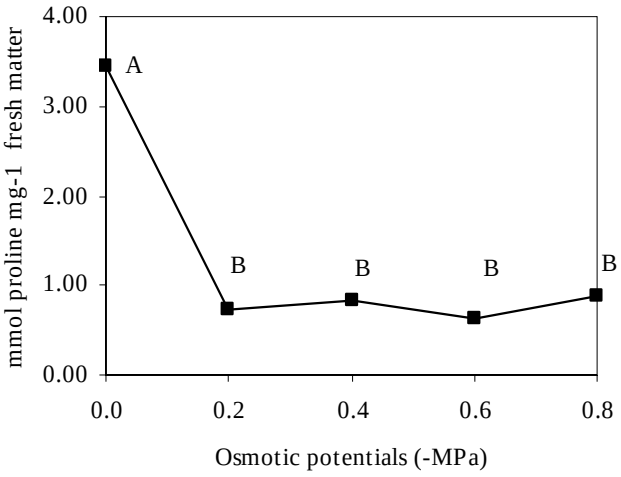

Figure-1. Proline levels in *Plantago ovata* seeds subjected to different osmotic potentials with the osmotic agents PEG, NaCl and $CaCl_2$.

There was no significant interaction between osmotic potentials and agents according to Tukey's test at 5% significance. Means regarding ascorbate peroxidase activity are presented in Table-1 and Figure-2. At -0.2 and -0.4 MPa there was no difference among osmotic agents, whereas -0.6 MPa PEG led to the highest ascorbate peroxidase activity, which significantly differed from those detected under NaCl solution. At -0.8 MPa, lower activity was observed under salt stress, compared to PEG-induced osmotic stress.

Oxidative stress, which is originated when plants are exposed to several types of environmental adversities, induces such plants to produce antioxidants against reactive oxygen species such as hydrogen peroxide, besides superoxide and hydroxyl radicals (Asada, 1994; Krause, 1994). Works performed by Gossett *et al*. (1994a, b) indicated higher ascorbate peroxidase activity under water stress, as well as temperature extremes (Spychalla & Desborough, 1990; Rainwater *et al*., 1996), mineral deficiency (Cakmak and Marschner, 1992) and treatment with herbicides (Harper and Harvey, 1978).

Table-1. Mean values of ascorbate peroxidase activity in *Plantago ovata* seeds subjected to different osmotic potentials and agents.

Osmotic potentials (-MPa)	**Osmotic agents**					
	PEG		**NaCl**		**$CaCl_2$**	
0	0.0449	ABa	0.0449	ABa	0.0449	ABa
0.2	0.0383	ABa	0.0547	Aa	0.0379	ABa
0.4	0.0299	Ba	0.0172	Ba	0.0190	ABa
0.6	0.0678	Aa	0.0338	ABb	0.0486	Aab
0.8	0.0538	ABa	0.0212	Bb	0.0162	Bb
CV (%) = 29.60	DMS = 0.0259					

Means followed by the same letter, uppercase in the column and lowercase in the line, did not differ according to Tukey's test at 5% significance.

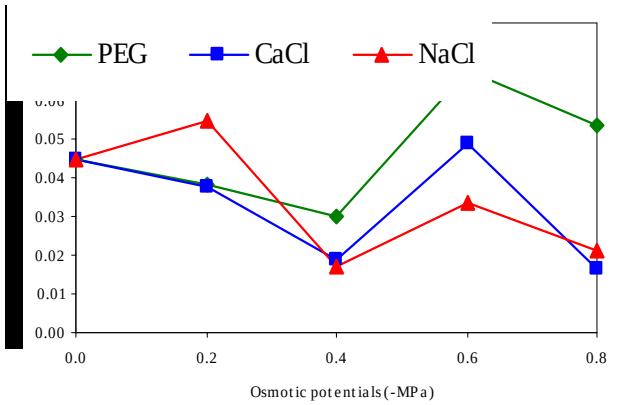

Figure-2. Ascorbate peroxidase activity in *Plantago ovata* seeds subjected to different osmotic potentials and agents.

The higher ascorbate peroxidase activity at -0.6 MPa was similar to that obtained by Baisak *et al*. (1994), who studied the effect of water stress induced by PEG on ascorbate peroxidase in wheat leaves and observed higher activity at -1.5 MPa; the same was detected by Zhang and Kirkham (1994), also in wheat leaves.

Amylase activity under different osmotic potentials and agents during *P. ovata* germination is presented in Table-2 and Figure-3. The water potentials induced by NaCl and $CaCl_2$ led to lower amylase activity than PEG. The latter increased enzymatic activity from -0.2 to -0.8 MPa, range in which such activity was constant (around 0.15mg starch min^{-1} mg^{-1} protein). Amylase activity did not significantly differ at potentials from -0.2MPa between both salt agents, which indicates that the absence of or delay in the germination due to the effect of different water potentials might have not been due to the amylase activity but to the seed vigor since amylase

activity was similar at potentials that led (-0.2 and -0.4 MPa) or not (-0.6 and -0.8 MPa) to germination.

Table-2. Mean values of amylase activity in *Plantago ovata* seeds subjected to different osmotic potentials and agents.

Osmotic potentials	Osmotic agents					
(-MPa)	PEG		NaCl		$CaCl_2$	
0	0.0755	Ba	0.0755	Aa	0.0755	Aa
0.2	0.1526	Aa	0.0344	Ab	0.0117	Bb
0.4	0.1528	Aa	0.0378	Ab	0.0118	Bb
0.6	0.1455	Aa	0.0366	Ab	0.0092	Bb
0.8	0.1612	Aa	0.0279	Ab	0.0104	Bb
CV (%) = 28.60	DMS = 0.0449					

Means followed by the same letter, uppercase in the column and lowercase in the line, did not differ according to Tukey's test at 5% significance.

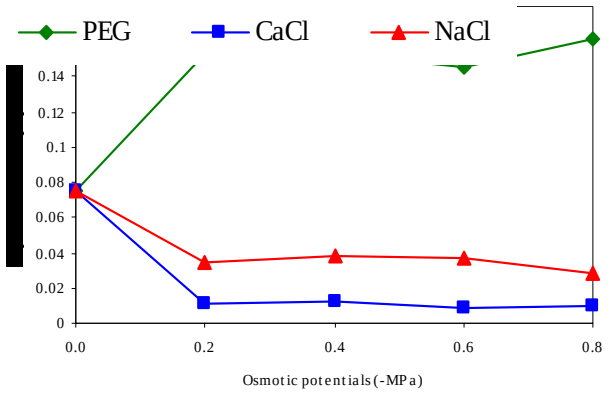

Figure-3. Specific activity of amylase in *Plantago ovata* seeds subjected to different osmotic potentials and agents.

Amylase activity was higher in -0.2 to -0.8 MPa PEG-treated seeds. The behavior under the remaining osmotic agents was different since there was no significant difference in NaCl-induced potentials and a significant reduction was detected under $CaCl_2$, which indicates that only the latter influenced amylase activity due to both its osmotic effect and toxic levels in the cells.

According to Larcher (2000), salt concentration is a stress factor for plants since its osmotic activity retains water and its ions act on the protoplasm. Water is osmotically retained in a salt solution; thus, the increase in salt concentration reduces water availability for the plant. The author also emphasizes that the adverse effects of salts may lead to functional damages and injuries. However, germination was not influenced by $CaCl_2$ in the present study (Table-3), although amylase behavior was affected.

Table-3. Germination percentage of *Plantago ovata* seeds subjected to different osmotic potentials and agents.

Osmotic potentials	Osmotic agents		
(-MPa)	PEG 6000	NaCl	$CaCl_2$
0	96.0 Aa	96.5 Aa	96.0 Aa
0.2	59.5 Bb	92.0 Aa	83.5 Aa
0.4	27.5 Cb	49.5 Ba	61.0 Ba
0.6	0.0 Da	0.0 Ca	0.0 Ca
0.8	0.0 Da	0.0 Ca	0.0 Ca

Means followed by the same letter, uppercase in the column and lowercase in the line, did not differ according to Tukey's test at 5% significance.

This suggests that a possible damage could have only occurred after germination since it seems not to be dependent on reserve mobilization or such mobilization might have occurred after germination, as observed by Oliveira Neto *et al.* (1998) in *Vigna unguiculata*.

CONCLUSIONS

Proline levels decreased during *Plantago ovata* germination under water and salt stress, whereas the activity of the enzymes ascorbate peroxidase and amylase was higher under stress induced by PEG than by NaCl or $CaCl_2$.

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