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β-galactosidases from cowpea stems: properties and gene expression under conditions of salt stress[1]

β-galactosidases de caules de feijão-de-corda: propriedades e expressão gênica em condições de estresse salino

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ABSTRACT - The aim of this work was to evaluate the properties and gene expression of isoforms of β-galactosidase, isolated from the stems of seedlings of the cowpea cv. Pitiuba, at different stages of development and sown in the absence and presence of NaCl. Seeds were sown onto sheets of filter paper soaked in distilled water (control) and a 100 mM NaCl solution. The plants were collected at the III (ED III) and VIII (ED VIII) stages of development. Isoforms of the enzyme β-gal I, II and III presented molecular weights of 89, 146 and 124 kDa respectively, and the same pH (4.0) and optimum test temperature (55 °C) under all the conditions studied. The three isoforms were stable up to 50 °C. The thermal inactivation curves of the enzymes as a function of time were biphasic at a temperature of 60 °C. Analysis of the gene expression of β-galactosidase was carried out using degenerate primers and total RNA from the stems of seedlings at ED III and ED VIII, sown both as control and under saline treatments. The results showed higher transcript levels at ED VIII, with reduced levels of β-galactosidase transcripts under saline treatment, and that β-gal I, II and III do not undergo changes during the early stages of development. There are a few differences in the properties of the three isoforms. Salinity affected the gene expression of enzymes. The enzymes were expressed differently for ED III and ED VIII.

Key words: *Vigna unguiculata*. Enzymes. Purification.

RESUMO - O objetivo foi avaliar as propriedades e a expressão gênica de isoformas de β-galactosidase isoladas de caules de plântulas de feijão-de-corda cv Pitiúba em diferentes estádios de desenvolvimento semeadas na ausência e presença de NaCl. As sementes foram semeadas em folhas de papel filtro embebidas em água destilada (controle) e em solução de NaCl 100 mM. As plântulas foram coletadas nos estádios de desenvolvimento III (ED III) e VIII (ED VIII). As isoformas da enzima, β-gal I, II e III, apresentaram massas moleculares de 89; 146 e 124 kDa, respectivamente, e o mesmo pH (4,0) e temperatura ótima de ensaio (55 °C), em todas as condições estudadas. As três isoformas mostraram-se estáveis até a temperatura de 50 °C. As curvas de termoinativação em função do tempo para as enzimas mostraram que, na temperatura de 60 °C, as curvas foram bifásicas. As análises da expressão gênica de β-galactosidases foram feitas usando *primers* degenerados e RNA total de caules de plântulas semeadas em condições controle e com tratamento salino, nos ED III e ED VIII. Os resultados revelaram maior nível de transcritos no ED VIII, com redução dos níveis de transcritos de β-galactosidases quando submetidos ao tratamento salino. Os resultados mostraram que as β-gal I, II e III não sofrem modificações ao longo dos estádios iniciais de desenvolvimento. Há algumas diferenças entre as propriedades das três isoformas. A salinidade afetou a expressão gênica das enzimas. As enzimas foram expressas diferentemente nos ED III e ED VIII.

Palavras-chave: *Vigna unguiculata*. Enzimas. Purificação.

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INTRODUCTION

Salinity is one of the main environmental factors limiting the growth and productivity of plants (PARIDA; DAS, 2005), which may respond differently when under this type of stress. These different plant responses to salinity may be related to the species or cultivars in question, to the stage of plant growth at the time of stress, to salt levels in the root environment, as well as to the duration of the stress (MUNNS, 2002).

When concentrations of salts in the cell cytosol increase the enzymatic activity involved in various metabolic pathways is inhibited (MUNNS, 2005). At the same time, various processes, such as protein synthesis, undergo changes caused by the ionic imbalance generated by the salt stress, which consequently lead to a reduction in plant growth (PARIDA; DAS, 2005).

Among the adaptations involved in the salinity tolerance of plants are certain molecular mechanisms (PARIDA, DAS, 2005). These however may be influenced by salt stress which has a strong effect on plant gene expression, with consequent changes in cellular machinery (ZHENG *et al*., 2006).

Although there is some information on the effects of salinity on enzymatic activity, such as that of the β-galactosidases isolated from cotyledons (ENÉAS-FILHO *et al*., 1995) and stems (SUDÉRIO *et al*., 2011a) of *Vigna unguiculata*, little is known about the effects of such stress on the physical and kinetic properties of these enzymes present in the cytosol and in the cell wall.

Studies into gene expression in seedlings of *Cicer arietinum* in relation to sowing time (two and eight days after sowing) revealed the presence of three different cDNAs of β-galactosidases associated with the cell wall of different plant organs (ESTEBAN; LABRADOR; DOPICO, 2005). In that work the seedlings were not subjected to any kind of stress, but from the results, the authors suggest that those β-galactosidases play an important role in the degradation of pectins in the cell wall of the plant organs and that the pattern of gene expression varies with the organ and the time of sowing. Other authors have also identified a type of cDNA corresponding to the gene which encodes a β-galactosidase involved in the degradation of pectins in the cell wall, but did not carry out any analysis which might relate it to stress. In the study, the researchers found that the expression of the gene in potato plants that had been genetically modified is related to a reduction in glycoside content and increased activity of the β-galactosidases (MARTÍN *et al*., 2005).

In work done by Sudério *et al*. (2011a) it was found that salt stress (100 mM NaCl) inhibited and delayed stem growth in cowpea seedlings, with this inhibition being more noticeable in the early stages of development. In addition, there was direct correlation between stem growth and the activity of galactosidase enzymes in the cell wall, especially in the early stages of development. However, it was not possible in this study to verify whether salinity directly influenced the activity of the galactosidases or the gene expression of these enzymes.

The aim therefore was to evaluate the kinetic properties and gene expression of β-galactosidases in the stems of cowpea seedlings at different stages of development, when sown with and without the presence of NaCl.

MATERIAL AND METHODS

Seeds of the cowpea [*Vigna unguiculata* (L.) Walp.] cv. Pitiúba were sown on sheets of filter paper soaked with distilled water (control) and with a saline solution (100 mM NaCl). Ten seeds were placed in a single row, approximately 1.5 cm from the top of the paper and 3.0 cm from the sides. After sowing, five sets of filter paper with seeds were individually rolled up and arranged vertically in 8 x 8 x 13 cm plastic containers holding 50 ml of the same solution used for moistening the paper. Four of these containers with paper rolls and seeds were then placed in 24 x 24 x 50 cm acrylic tanks, which were closed and kept at a photoperiod of 12h, under an irradiation of 16 μmol m^{-2} s^{-1}, a temperature of 25 ± 2 °C, with relative humidity close to 100%, for a period of approximately seven days for the control seedlings and eleven days for those under saline treatment. The greater the number of tanks employed, the greater the number of freeze-dried stems on which the process of extraction and purification of enzymes could be carried out. After this time the seedlings were selected in accordance with the classification described by Sousa *et al*. (2004) and collected at the (ED) III development stage, characterised by seedlings having radicles larger than 2 cm and smaller than 5cm, and at the (ED) VIII development stage, seedlings having radicles larger than 5 cm, cotyledons outside of the paper, a straight hypocotyl and open cotyledon leaves (SOUSA *et al*., 2004).

Protein extraction was carried out on freeze-dried stems in order to start the process of enzyme purification. The stems were macerated in a 25 mM sodium citrate buffer containing 50 mM potassium phosphate (pH 5.5) and 0.1% polyvinylpyrrolidone (PVP) at a ratio of 1:50 (w:v), with the macerate remaining in the buffer for one hour in an ice bath. The homogenate was centrifuged at 16,000 g for 30 minutes at 4 °C. The cytosolic protein fraction (F_{20-80}) was obtained from the supernatant (ENÉAS-FILHO *et al.* 1995). The precipitate was

resuspended and washed with a10 mM sodium phosphate buffer (pH 7.0) at 10 °C. The residue was then washed with acetone at -20 °C followed by washing with the same buffer. The filtrate was then discarded and the residue used for extraction of proteins present in the cell wall. To do this, the residue was resuspended in a buffer of 10 mM sodium citrate/10 mM sodium phosphate (pH 5.5) with 3 M NaCl and 0.1% (w:v) PVP at 4 °C for 24 hours under agitation. At the end of this time, the material was centrifuged at 16,000 g for 15 min at 4 °C. The precipitate was discarded and the supernatant filtered, dialysed against a buffer of 10 mM sodium citrate/10 mM sodium phosphate (pH 5.5) for 24 hours at 4 °C and centrifuged at 16,000 g for 15 min at 4 ºC. The precipitate was again discarded, with the supernatant corresponding to that fraction (F_{40-100}) of proteins ionically bound to the cell wall (SEARA; NICÓLAS; LABRADOR, 1988). The purity of the cell-wall preparation was tested, determining the possible presence of dehydrogenase enzymes of glucose 6-phosphate (cytosolic) (MOLINA *et al*., 2003), and vacuolar acid phosphatase (GRANJEIRO *et al*., 2003).

At each chromatography, 150,000 UA h^{-1} of the 20-80% fraction (F_{20-80} cytosolic proteins) were applied, freeze-dried and dissolved in a 5.0 mL buffer of Tris-HCl 25 mM (pH 7.2). The chromatography was carried out at a flow rate of 31.5 ml h^{-1} at 4 °C, with fractions of 4.2 mL being collected. The chromatographic profile was defined after reading the fractions at 280 nm and assessing β-galactosidase activity. The elution of the peaks retained on the column was done with a salt gradient (0.2-1.0 M NaCl in a buffer of 100 ml 25 mM Tris-HCl pH 7.2). (ENÉAS-FILHO *et al*., 2000).

The peaks with β-galactosidase activity which resulted from this ion exchange chromatography (with a minimum of three chromatographies) were pooled and dialysed against distilled water for 24 hours at 4 °C, separately concentrated by partial freeze-drying, and added to an affinity column (lactosyl-sepharose). During the purification of β-galactosidases present in the cell wall, the freeze-dried 40-100% fraction (F_{40-100} - proteins bound ionically to the cell wall) was directly added to the affinity column. The chromatography was carried out at a flow rate of 36.0 mL h^{-1} at 4 °C, with 4.8 mL fractions being collected. Elution was performed with an equilibration buffer containing 100 mM lactose and 0.5 M NaCl. Those fractions rich in β-galactosidase activity were pooled, dialysed against deionised water for 24 hours at 4 °C and stored at -20 °C (ENÉAS-FILHO *et al*., 2000).

The β-galactosidase activity was determined in accordance with Kanfer, Petrovich e Mumford(1973), with modifications and the determination of protein concentration done following the method described by Bradford (1976).

Molecular weight was determined by gel-filtration chromatography (Sephadex G-150). Enzymatic activity was studied for a pH range of from 2.5 to 6.5, in increments of 0.5. The activity test, to determine the optimal enzyme temperature, was performed at temperatures of 30, 40, 50, 55, 60, 70 and 80 °C. In order to determine the thermal stability of the β-galactosidases, 0.5 mL aliquots of the enzyme solution were incubated for 10 minutes at different temperatures (30, 40, 50, 60, 70 and 80 °C). Any thermal inactivation of the enzymes was verified by the pre-incubation of aliquots of 0.5 mL at 60 °C for 20, 40, 60 and 80 minutes, both with and without the presence galactose (50 mM) and glucose (50 mM). The values for enzyme activity were expressed as percentages of the controls (Control 1 - a sample with no heat treatment and without the presence of sugars; Control 2 - a sample with no heat treatment in the presence of 50 mM galactose; Control 3 - a sample with no heat treatment in the presence of 50mM glucose).

For the semi-quantitative RT-PCR analyses, β-galactosidase degenerate primers were designed from the alignment of cDNA previously used in phylogenetic studies (ESTEBAN; LABRADOR; DOPICO, 2005). These corresponded to the following species, with their respective GenBank accession numbers (http://www.ncbi.nlm.nih.gov): *Lupinus angustifolius* (AJ011047), *Vigna radiata* (AF229794), *Citrus sinensis* (AY029198), *Malus domestica* (L29451), *Pyrus pyrifolia* (AB0465543), *Lycopersicon esculetum* (AJ012798), *Carica papaya* (AF064786), *Arabdopsis thaliana* (NM125070), *Arabdopsis thaliana* 4 (AJ270300), *Arabdopsis thaliana* 3 (AJ270308) and *Arabdopsis thaliana* 2 (AJ270298). After alignment using Clustal X (THOMPSON *et al*., 1997), two well-conserved regions were used for designing the following degenerate primer pair: β-gal F: AGCACHCCHSARATGTGGC and β-gal R: 5' CGDGGHAYRTGRTACCATC 3'. The expected amplicon size was 1925 base pairs (bp).

Extraction of total RNA was carried out, isolated from 200 mg of the stems (previously stored in liquid N_2) of cowpea cv. Pitiúba seedlings at the III and VIII development stages, both under control conditions and saline tratment, using the "RNeasy Plant Mini Kit" (Qiagen) in accordance with the manufacturer's instructions. On-column DNase digestion was used to remove genomic DNA contamination.

The total isolated RNA was quantified by spectrophotometric reading at 260 nm (optical density of 1.0, corresponding to a concentration of 40 µg mL^{-1} total RNA) and the quality assessed by electrophoresis in agarose gel at 1.5% (integrity) and by the absorbance ratios A_{260}/A_{230} (to check for contamination by polysaccharides) and A_{260}/A_{280} (to check for contamination by proteins and/or phenols) (GASIC;

HERNANDEZ; KORBAN, 2004). The total RNA was then stored at -20 °C for later RT-PCR reaction.

Samples of total RNA were subjected to reverse transcription reaction. The test was conducted in 500 µl Eppendorf tubes using an Invitrogen "kit" and following the manufacturer's instructions. The reaction mixture contained 2 µg of total RNA, 4 µl of 5x Buffer (Invitrogen reaction buffer), 2 µl of 0.1M DTT, 2 µl of dNTP mix (each of 5 mM), 3µl Oligo dT_{18} l (20 pmol /µL) and DEPC water in sufficient quantity for 20 µl. This mixture was subjected to a 70 °C water bath for 10 minutes and then cooled on ice for two minutes to undo possible secondary structures of mRNA which might interfere with the reverse transcription reaction. The reverse transcription reaction was carried out at 37 °C for one hour and the reverse transcriptase was then inactivated by heating at 75 °C for 10 minutes. The samples were stored at -20 °C.

The products of the reverse transcription reaction (cDNA) and the degenerate primers were used for amplification of the β-galactosidase cDNA, by means of polymerase chain reaction (PCR). The PCR reactions were individually controlled by amplification of the actin cDNA (endogenous control) using specific primers obtained in accordance with Costa *et al.* (2004). The reaction mixture contained 1 µL RT product (cDNA), 1 µL 5 mM dNTPs, 5 µl 5x Buffer (250 mM Tris-HCl buffer, 250mM NaCl, 25 mM MgCl and 0.5 µM BSA), 1 µL primers for β-galactosidase genes [β-gal F (0.4 µM final), β-gal R (0.4 µM final)] or for the actin [actin F (0.4 µM final) actin R (0.4 µM final)], 0.15 µL taq polymerase (5 U/ µL) and sufficient sterile milli-Q water for 25 µL. This mixture was subjected to PCR cycles in a thermocycler [MJ Research, PTC-200 (Pertier Thermal Cycler)] using the following parameters: 95 °C for 3 minutes for initial DNA denaturation, followed by 30 cycles of denaturation (95 °C for 1 minute), annealing (55 °C for 1 minute) and extension (72 °C for 2 minutes). The PCR tests were performed following established parameters for estimating semi-quantitative transcript levels (CAVALCANTI *et al.*, 2013; COSTA *et al.*, 2010). Earlier tests enabled 30 cycles to be set as a number which was still outside the plateau region, and 55 °C as the optimal annealing temperature for both pairs of primers (β-galactosidase and actin). The PCR reactions were carried out in triplicate from a set of cDNAs (pooled sample).

The RT-PCR products were separated by electrophoresis in 1.5% agarose gel in a pH 8.0TBE buffer. Migration was performed using a Bio Rad adjustable DC power source (Power-pac 300) with a constant amperage of 50 mA for approximately 60 minutes at 25 °C. The gel was treated with ethidium bromide (0.5 µg mL^{-1}) for approximately 15 minutes and the bands corresponding to the cDNAs of the β-galactosidase and actin were detected by fluorescence emission obtained by exposing the gel to ultraviolet light. The gel was photographed using a gel imaging system (Labortechnik, Germany). Band density was analysed with the Scion Image beta 3 b release software (Scion Corporation, USA) (COSTA *et al.*, 2007). The expression data were normalised using the density ratio of the β-galactosidase cDNA bands to the actin (CAVALCANTI *et al.*, 2013; COSTA *et al.*, 2010). Specificity of the primers was verified by amplification of a single band having the expected sizes for the cDNA of β-galactosidase (1925 bp) and actin (530 bp).

RESULTS AND DISCUSSION

For all the conditions under study, the chromatographic profiles revealed the existence of two protein peaks (A_{280}), one being non-retained (DS I) and the other adsorbed to DEAE-Sephadex (DS II). Both DS I and DS II showed β-galactosidase activity. The DS I peaks showed a purification of 8.6, 36.5, 10.1 and 24.5 times for the F_{20-80} samples at stage III and VIII respectively (Table 1). The DS II peaks at the same stages of development and under the same treatments had a purification of 0.7, 1.3, 1.5 and 2.4 times respectively (Table 1).

Peaks with β-galactosidase activity, which were a result of the chromatography on DEAE-Sephadex A-50 (DS I and DS II), were subjected to affinity chromatography on lactosyl-sepharose. For all the conditions analysed, applying the DS I peaks to the affinity column resulted in a retained peak, named β-gal I, which demonstrated nearly all of the β-galactosidase activity. The retained peaks with β-galactosidase activity showed purification of 366.4, 42.8, 133.5 and 27.9 times at their respective development stages and treatments described above (Table 1).

The DS II peaks applied to the affinity chromatography, in a similar way to the DS I profile, also resulted in a retained peak that demonstrated nearly all the β-galactosidase activity, named β-gal II. This β-gal II was purified 30.3, 174.7, 21.4 and 102.4 times at ED III and ED VIII respectively (Table 1).

The 40-100% fractions (F_{40-100}), obtained from the extraction of proteins associated with the stem cell wall of cowpea seedlings at ED III and ED VIII, originating from seeds subjected to the control treatment and to the application of sodium chloride, were added to the lactosyl-sepharose affinity column.

For all the conditions under study, the chromatographic profiles revealed the existence of a retained-protein peak (A_{280}) with a considerable percentage of β-galactosidase activity in relation to the amount that was applied, named β-gal III. This enzyme at ED III was

Table 1 - Purification of cytosolic β-galactosidases and those present in the stem cell walls of cowpea seedlings [*Vigna unguiculata* (L.) Walp.] cv. Pitiúba at development stages III e VIII, subjected to salt stress

Development stage III					
Step	Activity (UA h^{-1})	Protein (mg P)	Specific activity (UA mg P^{-1} h^{-1})	Recupera-tion (%)	Purifica-tion (X)
Control (Distilled H_2O)					
DS-I	605,532	4.90	123,578	22.2	8.6
DS-II	881,867	82.29	10,716	32.3	0.7
β-gal I	262,933	0.05	5,258,660	9.6	366.4
β-gal II	283,073	0.65	435,496	10.3	30.3
β-gal III	174,062	0.23	756,791	33.3	49.0
Saline treatment (NaCl 100 mM)					
DS-I	496,905	0.96	517,610	18.8	36.5
DS-II	389,252	20.28	19,193	14.7	1.3
β-gal I	200,385	0.33	607,229	7.5	42.8
β-gal II	222,638	0.09	2,473,755	8.4	174.7
β-gal III	5,461	0.04	136,525	2.5	13.8
Development stage VIII					
Step	Activity (UA h^{-1})	Protein (mg P)	Specific activity (UA mg P^{-1} h^{-1})	Recupera-tion (%)	Purifica-tion (X)
Control (Distilled H_2O)					
DS-I	1,174,333	7.5	156,577	39.1	10.1
DS-II	744,785	31.5	23,643	24.8	1.5
β-gal I	453,696	0.22	2,062,255	15.1	133.5
β-gal II	245,180	0.74	331,324	8.1	21.4
β-gal III	59,418	0.06	990,300	28.4	52.3
Saline treatment (NaCl 100 mM)					
DS-I	621,209	1.1	564,736	22.8	24.5
DS-II	629,562	11.4	55,224	23.1	2.4
β-gal I	154,272	0.24	642,803	5.6	27.9
β-gal II	211,762	0.09	2,352,920	7.8	102.4
β-gal III	12,108	0.01	1,210,800	5.9	132.0

purified 49.0 times when subjected to the control treatment, and 13.8 times when subjected to salt stress. Whereas, at ED VIII it was purified 52.3 and 132.0 times under the control and saline treatments respectively (Table 1).

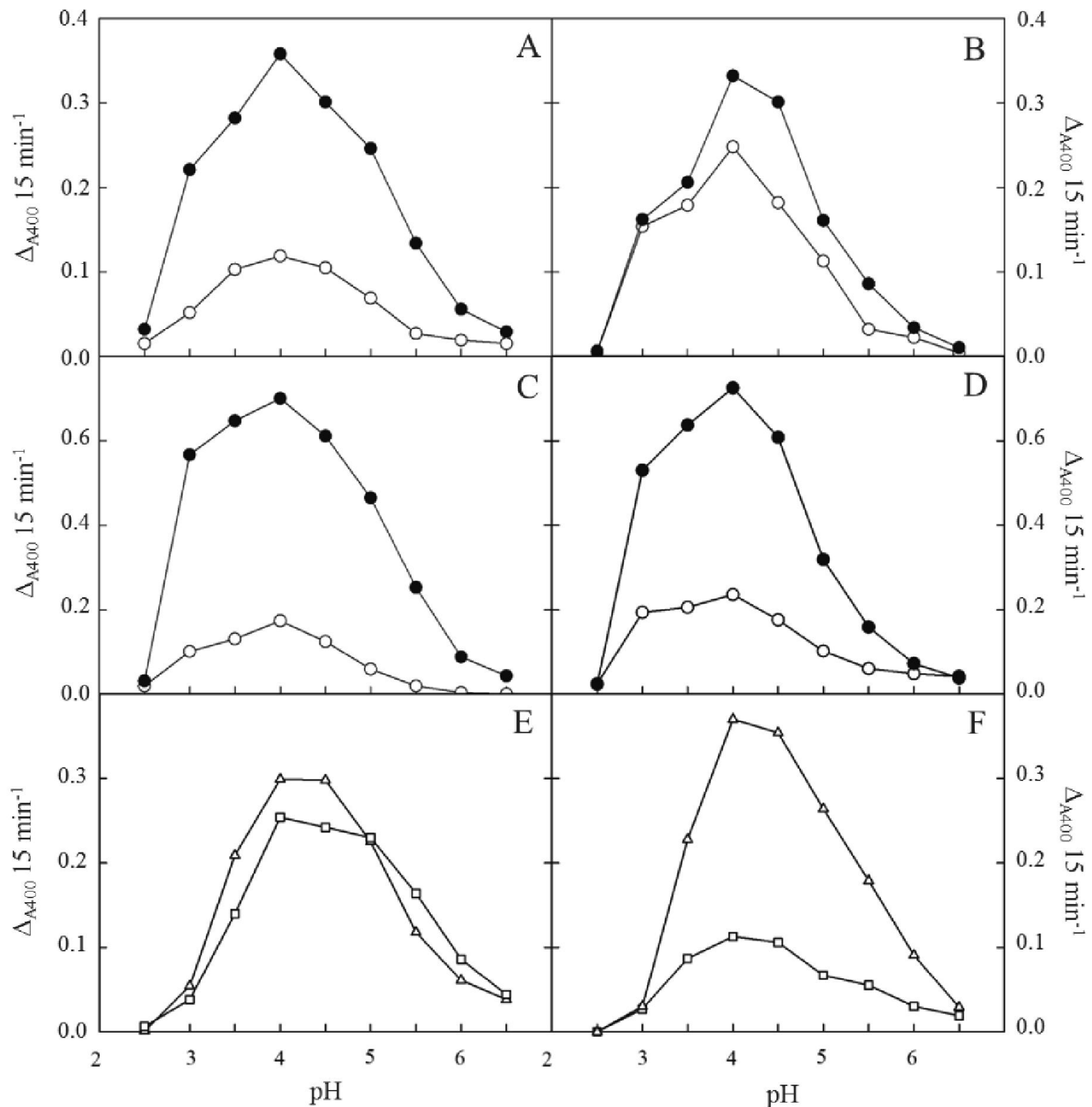
At ED III it was found that under conditions of salinity the seedlings showed a greater amount of protein relative to the control, however there was less β-gal I activity when the seedlings were subjected to saline treatment, showing that NaCl delays expression of the β-gal I enzyme. At ED VIII, it can be seen that treatment with sodium chloride also reduced β-gal I and II activity (AU h^{-1}) (Table 1). These results demonstrate that the β-gal I and II enzymes, both at ED III and ED VIII, display a reduction in activity which may be explained by possible changes taking place in the gene expression caused by the salinity.

The salinity also reduced the activity (UA h^{-1}) of β-gal III both at ED III and ED VIII. Together with this result there was a reduction in protein levels, indicating that the decrease in enzyme activity in the presence of NaCl may be related to the inhibition of protein synthesis in a similar way as that observed for the β-gal I and II isoforms.

In order to make a comparative analysis between the β-galactosidase at the two development stages (ED III and ED VIII) and subjected to the control or saline treatments, some enzymatic properties were determined. β-gal I, II and III showed apparent molecular weights of 89, 146 and 124 kDa respectively for all the conditions under study. In a similar way to the results found here, several studies with β-galactosidases purified from plants showed that isoforms of these enzymes present

diverse molecular weights (ALCÂNTARA *et al.*, 2006; BALASUBRAMANIAM *et al.*, 2005; BISWAS; KAYASTHA; SECKLER, 2003; SUDÉRIO *et al.*, 2011b). The activities of the cytosolic β-galactosidases (β-gal I and II) and cell wall β-galactosidase (β-gal III) as a function of the pH of the reaction medium, showed an increase from pH 2.5 until reaching maximum activity at pH 4.0, with a progressive decrease to pH 6.5 (Figure 1). The optimal pH activity range demonstrated by β-gal I, II and III is common to a wide variety of plant species, especially legumes (ALCÂNTARA *et al.*, 2006; BISWAS; KAYASTHA; SECKLER, 2003; CHILAKA; OKEKE; ADAIKPOH, 2002; ENÉAS-FILHO *et al.*, 2000; ENÉAS-FILHO *et al.*, 2001; SUDÉRIO *et al.*, 2011b). The optimum test temperature for the β-gal I, II and III enzymes was 55 °C, with a considerable drop in activity from 60 °C. The range seen for the optimum test temperature is

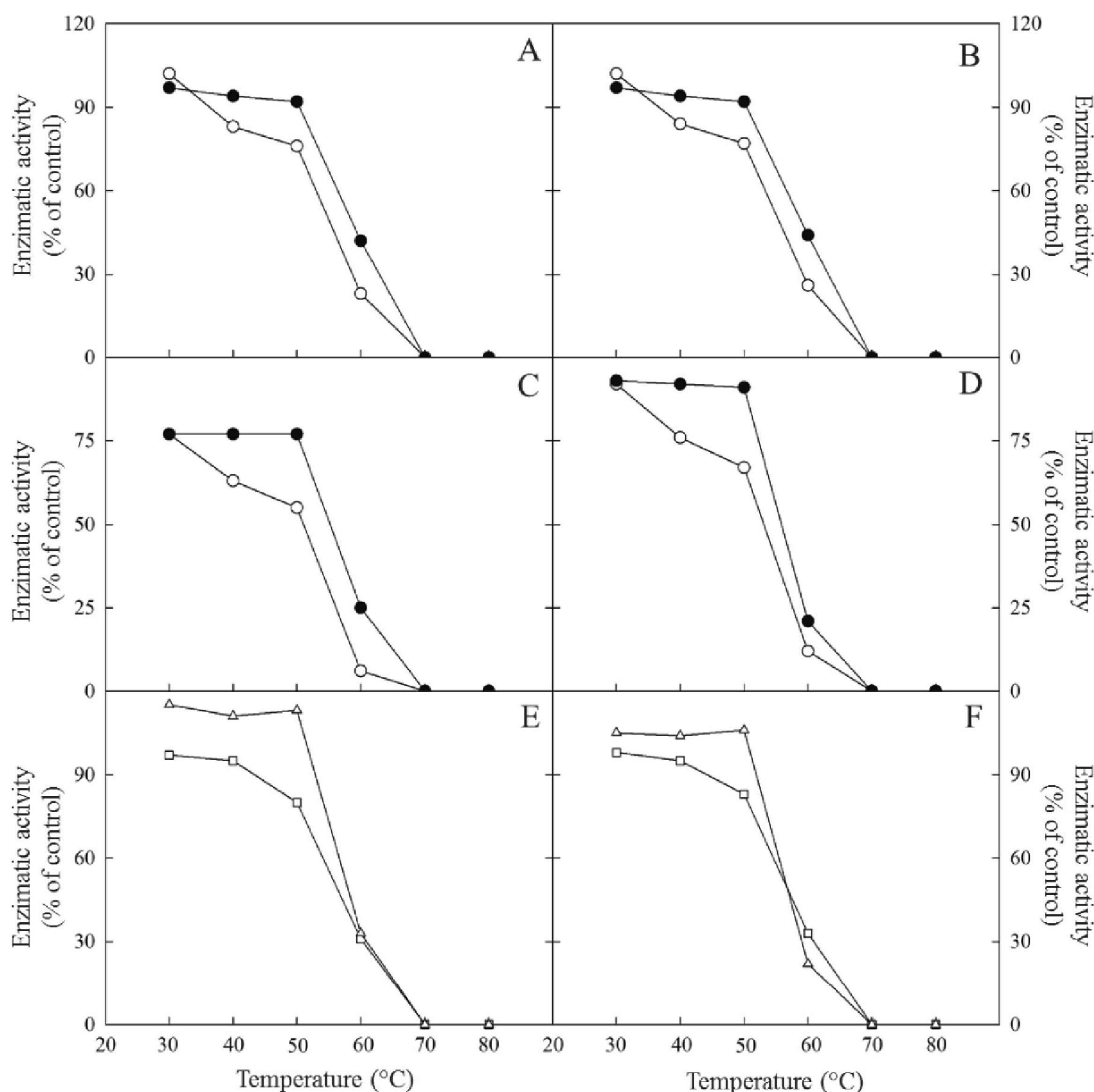
Figure 1 - Activity of the cytosolic β-galactosidases (β-gal I and β-gal II) and those present in the stem cell walls of cowpea seedlings [*Vigna unguiculata* (L.) Walp.] cv. Pitiúba (β-gal III) as a function of pH. Seedlings from the control (A, C and E) and saline treatment (B, D and F). **A e B -** development stage III (β-gal I, o—o; β-gal II, •—•); **C e D -** development stage VIII (β-gal I, o—o; β-gal II, •—•); **E e F** - β-gal III (development stage III, □—□; development stage VIII, Δ—Δ)



similar to those found for other β-galactosidases purified from plants (CHILAKA; OKEKE; ADAIKPOH, 2002; ENÉAS-FILHO *et al*., 2000; ENÉAS-FILHO, 2001; SUDÉRIO *et al*., 2011b). In the study of thermostability, β-gal I showed a reduction in activity when incubated at just 40 °C, with all enzymes showing a sharp drop in activity at 60 °C until complete inactivation at 70 °C (Figure 2).

In general, thermal inactivation as a function of time for β-gal I and II (Figures 3 and 4) in the absence of any sugars (control) displayed biphasic curves, having a rapid initial phase with loss of activity, and a slower second phase. The β-gal I and II enzymes lost all activity when pre-incubated at 60 °C, but only after 80 minutes of pre-incubation. The protective effect of glucose and galactose against the thermal inactivation

Figure 2 - Activity of the cytosolic β-galactosidases (β-gal I and β-gal II) and those present in the stem cell walls of cowpea seedlings [*Vigna unguiculata* (L.) Walp.] cv. Pitiúba (β-gal III) as a function of pre-incubation temperature. Seedlings from the control (A, C and E) and saline treatment (B, D and F). **A e B -** development stage III (β-gal I, o—o; β-gal II, •—•); **C e D -** development stage VIII (β-gal I, o—o; β-gal II, •—•); **E e F** - β-gal III (development stage III, □—□; development stage VIII, Δ—Δ)



of β-gal I and II was also found (Figures 3 and 4), with the loss of activity being less in the presence of galactose than in the presence of glucose.

The biphasic thermal inactivation curve may reflect the ability of the enzyme molecule to exist in more than one stable conformational state (DANIEL; DINES; PETACH, 1996). The stabilisation of β-gal I and II against thermal inactivation in the presence of glucose and galactose suggests that the pathway involves changes to the active site of the enzymes, in such a way that exposure to high temperatures may not necessarily inactivate them due to the protection of these sugars, which probably bind to the enzymes, changing their conformations and turning them more resistant to high temperatures (CHILAKA; OKEKE; ADAIKPOH, 2002). Studies into thermal inactivation conducted on β-galactosidase in the peanut (*Kestingella geocarpa*) (CHILAKA; OKEKE; ADAIKPOH, 2002) and cowpea (*Vigna unguiculata*) (SUDÉRIO *et al.*, 2011b) showed similar results to those found in this study, in as much as the glucose and galactose also protected the β-galactosidases obtained from *K. geocarpa* against thermal inactivation. As could be seen, there was practically no significant difference between the properties of the β-galactosidases when extracted from the stems of seedling which were subjected to saline treatment at different development stages (ED III and ED VIII).

Analysis was then carried out of the gene expression of β-galactosidases from the stems of cowpea seedlings (Figure 5A). The highest level of transcripts was detected at ED VIII, with seedlings from the control treatment showing an amount of β-galactosidase transcripts at least 50% greater than seedlings subjected to salt stress (Figure 5B).

The quantity of transcripts detected at ED III, both for the control and saline treatments, was very low. These results indicate that in young seedlings, such as those collected at ED III, gene expression did not occur with the same intensity as in seedlings collected at ED VIII. Furthermore, it was possible to infer that salinity influences the gene expression of the β-galactosidase, agreeing with the results obtained in the purification of

Figure 3 - Thermal inactivation of β-gal I from the stems of cowpea seedlings [*Vigna unguiculata* (L.) Walp.] cv. Pitiúba under control conditions (A, C) and saline treatment (B, D), in the absence (o—o) and presence of 50 mM glucose (▲—▲) and 50 mM galactose (■—■). **A. and B.** Development stage III; **C. and D.** Development stage VIII

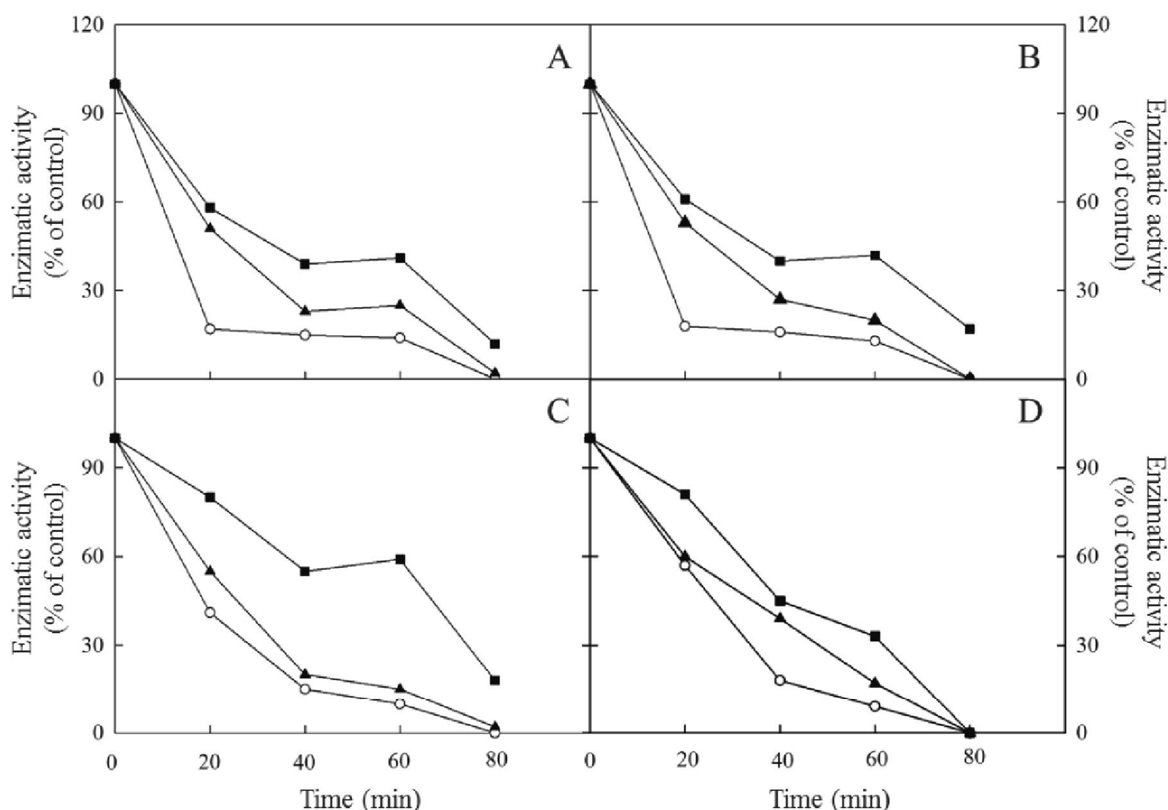


Figure 4 - Thermal inactivation of β-gal II from the stems of cowpea seedlings [*Vigna unguiculata* (L.) Walp.] cv. Pitiúba under control conditions (A, C) and saline treatment (B, D), in the absence (o—o) and presence of 50 mM glucose (▲—▲) and 50 mM galactose (■—■). **A. e B.** Development stage III; **C. e D.** Development stage VIII

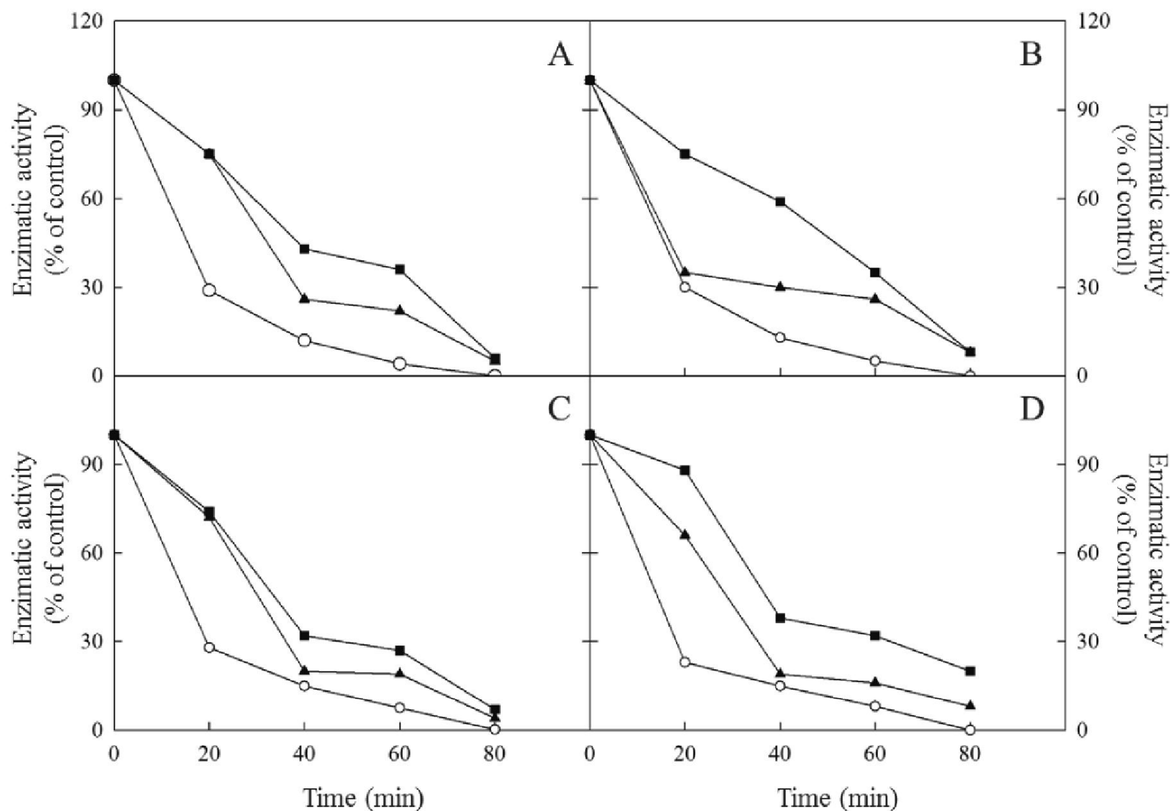
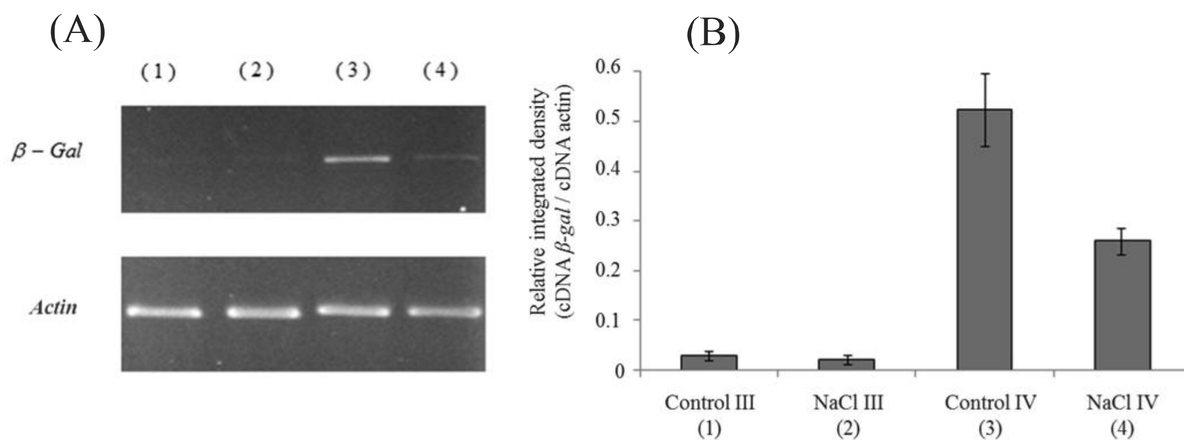


Figure 5 - Analysis of β-galactosidase transcripts from the stems of cowpea seedlings [*Vigna unguiculata* (L.) Walp.] cv. Pitiúba. A - RT-PCR products of β-galactosidase and Actin (endogenous control) visualised on 1.5% agarose gel; B - Normalisation of the quantity of β-galactosidase transcripts using the ratio of integrated densities of the β-galactosidase cDNA bands to the actin. The data (B) are the averages ± SD of three independent reactions/gels from a set of total RNA (pooled sample). 1-development stage III (control); 2- development stage III (saline treatment); 3- development Stage VIII (control); 4- development Stage VIII (saline treatment)



these enzymes (Table 1). With respect to the analysis of gene expression as a function of the physiological age of the seedlings, those results are in agreement with those found by Esteban, Labrador e Dopico (2005) who, although not analysing gene expression patterns under salt stress, did analyse the temporal expression of cDNAs responsible for encoding β-galactosidases associated with the cell wall of the epicotyl, mesocotyl and roots of *Cicer arietinum* seedlings at two and eight days after sowing.

CONCLUSIONS

The β-gal I, II and III enzymes do not undergo any changes in their physical and kinetic properties regardless of development stage, or the absence or presence of salinity. However, salinity does negatively influence β-galactosidase gene expression, which is more intense in stems of seedlings at ED VIII, when compared to seedlings at ED III.

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