SEMINA
Ciências Agrárias

DOI: 10.5433/1679-0359.2021v42n1p419

Enzymatic activity in azuki bean seedlings subjected to salinity and water deficiency

Atividade enzimática em plântulas de feijão-azuki submetidas a salinidade e a restrição hídrica

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Highlights

Water stress promotes an increase in protein content in azuki bean seedlings.
Excess sodium in the soil solution decreases enzyme activity.
Potassium increases the production of antioxidant enzymes after salt stress.

Abstract

Salinity and water deficiency are factors that limit the initial development of crops, directly interfering with the efficiency of food production. Studies on the behavior of cultivable species under stress are important to determine management actions; therefore, the mechanisms involved in post-stress recovery should be investigated. The objective of this study was to evaluate the effect of water and salt stress on enzymatic activity in azuki bean seedlings. The experimental design was a completely randomized, 4 × 6 factorial arrangement (four reagents: $CaCl_2$, KCl, NaCl, and polyethylene glycol 6000 (PEG 6000) × six osmotic potentials: 0.0, -0.2, -0.4, -0.8, -1.2, and -1.6 MPa). The quantification of protein content and analysis of enzyme (catalase, peroxidase, and phenylalanine ammonia-lyase) activity in seedlings were performed on the 10th day after sowing. NaCl is toxic and recovery of azuki bean seedlings was less likely under salt stress owing to reduced enzymatic activity. In contrast, seedlings subjected to KCl treatment showed increased production of antioxidant enzymes.

Key words: Antioxidant enzymes. *Vigna angularis* L. Water stress. Salinity stress.

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Received: Oct. 03, 2019 - Approved: Oct. 27, 2020

Resumo

A salinidade e a restrição hídrica são fatores limitantes ao desenvolvimento inicial das culturas, interferindo diretamente na eficiência da produção de alimentos. Estudos sobre o comportamento das espécies cultiváveis sob estresse são importantes para determinar ações de manejo, por essa razão deve haver a investigação e compreensão dos mecanismos envolvidos na recuperação pós estresse. O objetivo do trabalho foi avaliar o efeito do estresse hídrico e salino sobre a atividade enzimática de plântulas feijão-azuki. O delineamento experimental utilizado foi o inteiramente casualizado, em arranjo fatorial 4x6 (4 reagentes: $CaCl_2$, KCl, NaCl e polietilenoglicol 6000 (PEG 6000) x 6 potenciais osmóticos 0,0; -0,2; -0,4; -0,8; -1,2; -1,6 MPa). A quantificação do teor de proteínas e atividade das enzimas catalase, peroxidase e fenilalanina amônia-liase de plântulas foram analisadas no 10º dia após semeadura. O NaCl é tóxico e possibilita menores chances de recuperação em plântulas de feijão-azuki sob estresse salino devido a menor atividade enzimática. Por outro lado, as plântulas submetidas à solução de KCl apresentam aumento da produção de enzimas antioxidantes.

Palavras-chave: Enzimas antioxidantes. *Vigna angularis* L. Estresse hídrico. Estresse salino.

Azuki beans (*Vigna angularis* (Willd.) Ohwi & Ohashi) are traditionally grown in Asian countries, and their use is associated with the food industry. The benefits of eating pulses are the provision of up to three times more protein than any cereal, in addition to the supply of other functional components. Awareness regarding these advantages has grown among populations and adherence to a healthy diet has gradually increased consumption (Liu et al., 2016).

Plants are susceptible to biotic and abiotic stresses. Among the limitations on the initial development of crops, salt stress and water deficit stand out; these interfere in metabolic processes and change the physiological and biochemical characteristics of cultivated plants (Złotek, Szymanowska, Baraniak, & Karaś, 2015). To overcome stress, plants have the ability to modulate metabolic responses in order to return to the usual survival metabolism. When suffering oxidative stress, plants stimulate biosynthesis and intensify the activity of antioxidant enzymes, which is an important cellular signal for reactions of the plant defense system (Soares & Machado, 2007). The increase in antioxidant enzymes reduces oxidative damage as the production and accumulation of reactive oxygen species, i.e., oxygen ions, peroxides, and free radicals, is obstructed (Zlatev & Lidon, 2012).

According to Ahmed et al. (2014), studies that combine salt and water stress are of practical and ecological importance. When the limitations are at extreme levels, the success of cultivation is drastically reduced; therefore, to ensure adequate and targeted planning, it is essential to know the behavior of the genotype of interest in adverse conditions. Based on the above considerations, the objective of this study was to evaluate the effect of water and salt stress on the enzymatic activity of azuki bean seedlings.

The experiment was conducted under laboratory conditions at Londrina-PR. A completely randomized design was adopted, with four replications per treatment, arranged in a 4 × 6 factorial arrangement. The effects of water and salt stress on the enzymatic activity

of azuki bean seedlings were evaluated by preparing four solutions: $CaCl_2$, KCl, NaCl, and polyethylene glycol (PEG) 6000 adjusted to six osmotic potentials: 0.0, -0.2, -0.4, -0.8, -1.2, and -1.6 MPa.

The concentrations were obtained based on the Van't Hoff equation, i.e., $Y_{os} = -RTC$, where Y_{os}: osmotic potential (atm), R: universal gas constant (0.082 atm L mol^{-1} K), T: temperature (K), and C: concentration (mol L^{-1}) of treatment solutions as described by Villela, Doni and Sequeira (1991).

Four subsamples of 50 azuki bean seeds each, belonging to the phytotechnology seed bank of the State University of Londrina, were sown for germination between three sheets of paper moistened with the solutions to be tested, in the proportion of 2.5 times the mass of the dry paper. The rolls were kept in a Mangelsdorf-type germinator regulated at a temperature of 25 °C for 10 days.

Each sample comprised 1 g of plant material (whole seedlings, except cotyledons) macerated in 20 mL of phosphate buffer (0.2 M and pH 7.5), and subsequently centrifuged at 12,000 (×g) at a temperature of 4 °C for 10 min. All analyses were performed in triplicate (Bertoncelli, Alamino, Oliveira, Marchesan, & Loss, 2015).

Total protein was determined using the methodology proposed by Bradford (1976). The activity of the enzyme phenylalanine ammonia-lyase (PAL-E.C. 4.3.1.5) was evaluated using the methodology proposed by Umesha (2006) with some modifications. Seedlings (200 mg) were macerated in 2 mL of pH 6 phosphate buffer, and then centrifuged at 6,000 (×g) at a temperature of 4 °C for 10 min. Four hundred (µL) of the supernatant fractions were removed and placed in tubes containing 800 µL of TRIS-HCl buffer (0,5 M, pH 8.0) and 800 µL L-phenylalanine (6 µM). Subsequently, the contents of the tubes were homogenized and incubated at 37 °C for 60 min. The reaction was stopped by adding 100 µL of 5 N HCl. Absorption readings were performed at 290 nm using a spectrophotometer Micronal AJX-1600. The enzymatic activity of PAL was expressed in µg equivalents of trans-cinnamic acid min^{-1} mg^{-1} of protein. Catalase (CAT) was determined by evaluating samples prepared with a solution containing 900 µL of potassium phosphate buffer solution (0.05 M and pH 7.0) and hydrogen peroxide (12.5 mM), added to 100 µL supernatant protein extract. Absorption was measured at 240 nm and a molar extinction of 36 mM^{-1} cm^{-1} using a spectrophotometer (Anderson, Prasad, & Stewart, 1995).

Peroxidase activity (POD) was quantified by converting guaiacol to tetraguaiacol by adding 900 µL of solution containing 250 µL of guaiacol, 306 µL of hydrogen peroxide, and potassium phosphate buffer solution (0.01 M and pH 6.0 to 100 mL) in 100 µL of supernatant protein extract. Data were recorded at 470 nm using a spectrophotometer (Lusso & Paschoalati, 1999).

The results were subjected to an analysis of variance (F-test). The treatment averages were compared using the Tukey test ($p \leq 0.05$) for the qualitative factor (products) and the quantitative factor (osmotic potentials) was adjusted by orthogonal polynomials up to the second order using the statistical software R version 3.2.0.

Analysis of variance revealed that the interaction between the products and osmotic potentials was significant at the 5% probability of error level with the F-test for all evaluated characteristics.

Protein content was high when the PEG 6000 treatment was applied with osmotic potentials of -0.2, -0.4, and -0.8 MPa, and at the last two potential levels, values did not differ from those of the NaCl and $CaCl_2$ treatments. At potential levels of -1.2 and -1.6 MPa, the highest protein content was with the $CaCl_2$ and KCl treatments, respectively. Except for the osmotic potential of -0.4 MPa, the lowest levels were obtained when seedlings were subjected to NaCl treatment (Table 1).

The highest concentrations of the CAT enzyme were obtained with $CaCl_2$ treatment at the potential of -0.2 MPa, with PEG 6000 at -0.4 and -0.8 MPa, and with KCl at -1.2 and -1.6 MPa. In contrast, treatment with NaCl obtained the lowest averages at all potential levels, with the exception of -0.2 MPa, not differing from PEG 6000 at -1.2 MPa and $CaCl_2$, KCl, and PEG 6000 at -1.6 MPa. For the enzymes POD and PAL, the highest levels were found when KCl was used, regardless of the osmotic potential (Table 1).

Only the treatment with PEG 6000 increased protein content compared to that in the control (0.0 MPa), with a maximum response point at a potential of -0.31 MPa with protein values of 9.89 mg g^{-1}. In contrast, when the seedlings were in contact with NaCl, the activities were not significant ($p > 0.05$) from -1.2 MPa (Figure 1).

For all enzymes, there was no significant ($p > 0.05$) adjustment to the regression analysis for PEG 6000; in the NaCl treatment, only POD was not significant. The CAT, POD, and PAL activities were intensified to the point of maximum response at potentials of -0.62, -0.68, and -0.18 MPa, respectively, for $CaCl_2$ and -2.41, -0.97, and -0.93 MPa, respectively, for KCl (Figure 1).

Table 1
Protein content and enzymatic activity of catalase (CAT), peroxidase (POD), and phenylalanine ammonia-lyase in azuki bean seedlings in contact with $CaCl_2$, KCl, NaCl, and polyethylene glycol (PEG) 6000 solutions at different osmotic potentials. Londrina, PR

Products	Osmotic potential (MPa)						
	0.0	-0.2	-0.4	-0.8	-1.2	-1.6	
	Protein (mg g^{-1} tissue)						C.V (%)
$CaCl_2$	8.58**	7.63 B*	6.63 B	11.00 A	11.45 A	0.00 B	14.57
KCl		4.20 C	4.24C	4.27 B	4.38 B	5.19 A	
NaCl		7.19 B	11.77 A	2.00 C	0.00 C	0.00 B	
PEG 6000		9.50 A	10.55 A	11.10 A	0.00 C	0.00 B	
	CAT (mmol H_2O_2 mg^{-1}protein)						C.V (%)
$CaCl_2$	0.26**	0.33 A	0.44 B	0.35 C	0.32 B	0.00 B	13.33
KCl		0.21 C	0.49 B	0.57 B	0.78 A	0.82 A	
NaCl		0.28 AB	0.35 C	0.04 D	0.00 C	0.00 B	
PEG 6000		0.24 BC	0.78 A	1.10 A	0.00 C	0.00 B	
	POD (UAbs min^{-1} mg^{-1}protein)						C.V (%)
$CaCl_2$	0.04**	0.27 C	0.31 B	0.25 B	0.13 B	0.00 B	15.65
KCl		0.48 A	0.49 A	0.51 A	0.57 A	0.45 A	
NaCl		0.33 B	0.21 C	0.07 C	0.00 C	0.00 B	
PEG 6000		0.25 C	0.24 C	0.25 B	0.00 C	0.00 B	
	PAL (UAbs min^{-1} mg^{-1}protein)						C.V (%)
$CaCl_2$	0.14**	0.12 B	0.12 C	0.10 C	0.09 B	0.00 B	17.54
KCl		0.19 A	0.23 A	0.24 A	0.27 A	0.19 A	
NaCl		0.11 B	0.09 D	0.02 D	0.00 C	0.00 B	
PEG 6000		0.11 B	0.18 B	0.21 B	0.00 C	0.00 B	

* Within column, means followed by a different letter differ significantly at 5% probability level by Tukey's Test.
** Average corresponding to the osmotic potential 0.0 MPa for all products.

For protein content, the enzymatic activity of CAT and PAL was lower when the seedlings were in contact with NaCl than that with the other treatments; the activities at potentials above -1.2 MPa were negligible (Figure 1).

Plants under stress produce reactive oxygen species in chloroplasts and mitochondria, i.e., superoxide radicals, hydrogen peroxides, and hydroxyl radicals, which act together or in isolation and catalyze oxidative damage that compromises the normal functioning of cells and plant growth (Soares & Machado, 2007).

Plant defense mechanisms stimulate the biosynthesis and activity of non-enzymatic and enzymatic products with antioxidant power, such as CAT, POD, and PAL, to check oxidative stress (Farooq, Hussain, Walker, & Siddique, 2015). This detoxification ability is essential and determined by morphological, physiological, and biochemical factors that

have the objective of maintaining homeostasis and therefore, modulate the entry of salts through the roots and the production of osmoprotectors (H. Abbasi et al., 2016).

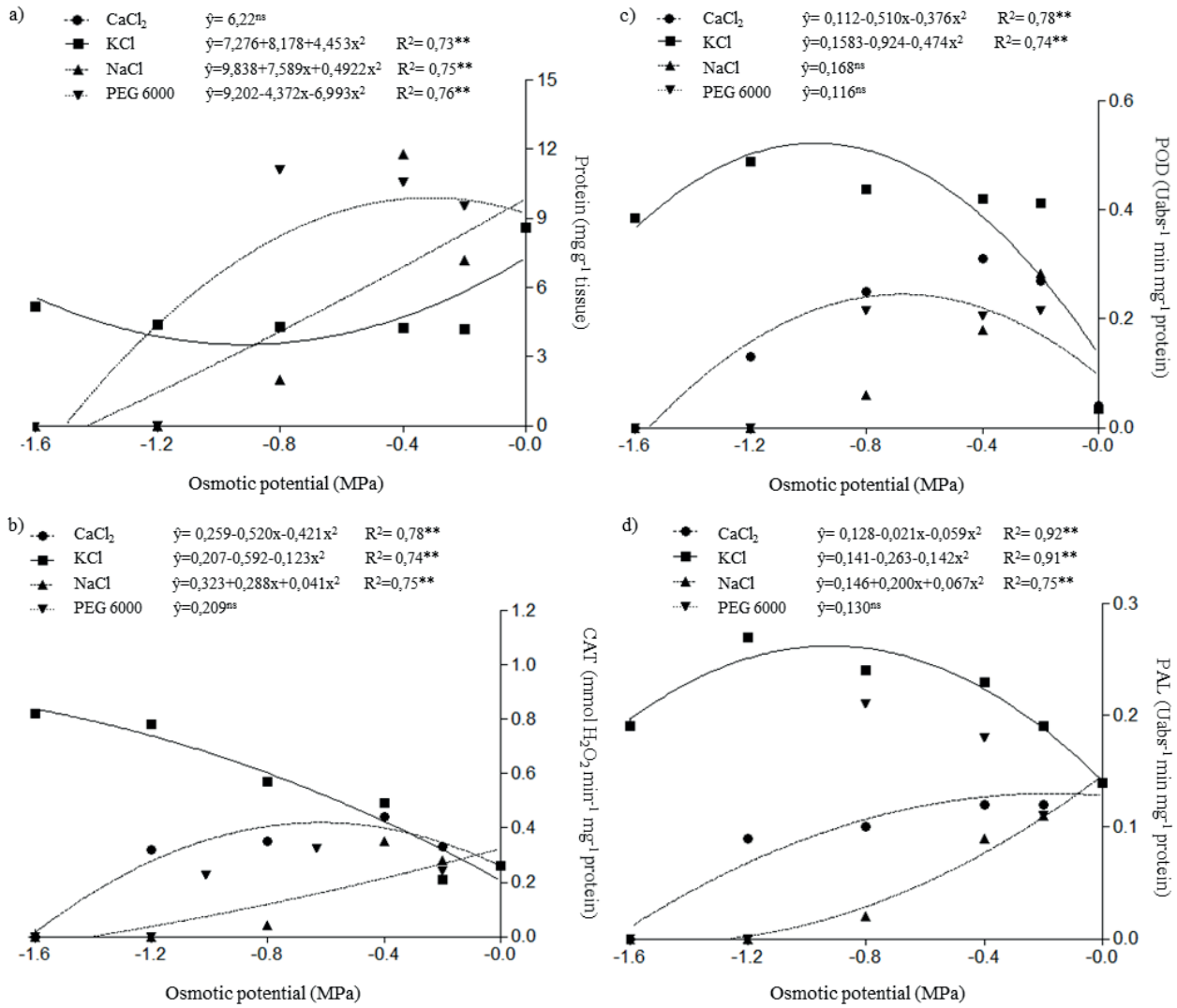

Figure 1. Protein content (a) and catalase (CAT) (b), peroxidase (POD) (c) and phenylalanine ammonia-lyase (PAL) (d) enzyme activity in azuki bean seedlings treated with $CaCl_2$, KCl, NaCl, and PEG 6000 solutions at different osmotic potentials.

The activities of these enzymes increase during stress and are important to enable detoxification to mitigate the effects of oxidative stress (Zheng et al., 2008). The increase in activity and production of these enzymes could likely facilitate osmotic rebalancing in the presence of water and salt stress and the plant's recovery after hydration (Ahmed et al., 2014).

Water deficiency impairs the absorption and translocation of nutrients, and salinity promotes nutritional imbalance because NaCl

is more soluble and abundant and competes with other ions, such as potassium, calcium, and nitrate (Munns & Tester, 2008). Both stresses cause cell dehydration, damage to the membrane, reduced activity and denaturation of the cytosol and organelle proteins, and depending on severity, they cause the breakdown of cell metabolism (Mahajan & Tuteja, 2005). This may explain the low values obtained in this study for protein and enzyme response levels when the interaction between the NaCl reagent and osmotic potential was investigated.

The relationship between sodium and potassium is critical for stress tolerance; the former competes directly with the latter for enzymatic sites, causing deficiency, and osmotic and water imbalance (Akham et al., 2010). Working on the tolerance of corn hybrids under salt stress, G. H. Abbasi et al. (2014), found that the tolerant varieties had a higher K^{+}/Na^{2+} ratio than the sensitive ones; this implies the accumulation of potassium, an essential nutrient for the promotion of cell turgidity, because of the potentiation of water absorption by plants. These facts support the results obtained in the present study, in which the use of KCl enabled higher protein and enzyme content at the most negative osmotic potentials than those with the other solutions.

The relationship between the maximum limit of tolerated sodium and the imbalance of the Na/K, Na/Ca, and Na/Mg ratios reduces plant growth and crop yield (Santos, Ruiz, Neves, Freire, & Freire, 2009). Studies have shown that salt-tolerant cultivars tend to accumulate more potassium ions, and this condition increases the likelihood of survival of plants under stress. Therefore, adding this element to the saline solution could improve the growth of the aerial part and roots, potentiate the activity of antioxidant enzymes, and reduce the amounts of malondialdehyde, soluble sugars, and the electrolytic leakage in salt-sensitive cultivars (Zheng et al., 2008).

Su et al. (2012) found that saline stress containing silicon, potassium, and calcium ions increased the activity of the enzymes superoxide dismutase, CAT, and POD. Consequently, there was a reduction in oxidative cell damage and improvement in the recovery capacity of plants after rehydration when compared to a solution containing NaCl.

In addition to the elimination of free radicals through enzymatic systems, plant metabolism has an effective mechanism for drought tolerance, i.e., the expression of stress-inducible genes that code and produce regulatory proteins, kinases, phosphatases, and aquaporins capable of minimizing the effects cellular dehydration (Krasensky & Jonak, 2012). Therefore, the water stress caused by PEG 6000 triggered the increase in protein production.

The toxic nature of NaCl makes recovery of azuki bean seedlings less likely under salt stress owing to reduced enzymatic activity. In contrast, seedlings subjected to KCl treatment exhibited increased production of antioxidant enzymes.

Acknowledgment

We are grateful to the Coordination for the Improvement of Higher Education Personnel (CAPES) for the postgraduate scholarships granted and to the Araucária Foundation for the Productivity Scholarship in Research granted to Takahashi, L. S. A.

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