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Effects of ascorbic acid on the germination and vigour of cowpea seeds under water stress[1]

Efeitos do ácido ascórbico na germinação e vigor de sementes de feijão-caupi sob estresse hídrico

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ABSTRACT - Water is one of the most compromising factors in the germination and initial growth of seedlings, where its restriction causes a reduction in the water potential of cells in addition to causing oxidative stress. Ascorbic acid (AsA) is known to protect organelles and cells against the accumulation of ROS. The aim of this work was to study the effects of ascorbic acid on the conditioning of cowpea seeds subjected to water stress. The seeds of the BRS Marataoã and Setentão genotypes were conditioned at a concentration of 0.0 (control), 0.25, 0.50, 0.75 and 1.00 mM AsA, and sown on paper rolls (Germitest®) moistened with mannitol solution at a potential of 0.0 (control), -0.3, -0.6, -0.9 and -1.2 MPa, and stored in a germination chamber (BOD) at 25 ºC. The experiment was conducted in a completely randomised design, in a 2×5×5 factorial scheme, with four replications of 50 seeds per treatment. The variables under analysis were percentage germination, first germination count, germination speed index, shoot and root length, total seedling dry weight and electrolyte leakage in the leaves and roots. Conditioning the seeds with ascorbic acid at a concentration of 0.50 mM for the BRS Marataoã genotype and 0.75 mM for the Setentão genotype, enabled the development of more vigorous seedlings and a reduction in the damage caused to the membranes by oxidative stress, both in the absence of a water deficit and at the osmotic potentials under test, including at the lowest potential.

Key words: *Vigna unguiculata* L.. Oxidative stress. Drought.

RESUMO - A água é um dos fatores que mais compromete a germinação e o crescimento inicial das plântulas, com sua restrição provocando reduções no potencial hídrico da célula, além de causar o estresse oxidativo. O ácido ascórbico (AsA) é conhecido por proteger organelas e células contra as EROs evitando seu acúmulo. Objetivou-se estudar os efeitos do ácido ascórbico no condicionamento de sementes de feijão-caupi submetidos ao estresse hídrico. As sementes dos genótipos BRS Marataoã e Setentão foram condicionadas nas concentrações 0,0 (controle); 0,25; 0,50; 0,75 e 1,00 mM de AsA e semeadas em rolos de papel (Germitest®), umedecidos com soluções de manitol nos potenciais de 0,0 (controle); -0,3; -0,6; -0,9 e -1,2 MPa e acondicionados em câmara de germinação (B.O.D.) na temperatura de 25 ºC. Conduzido em delineamento inteiramente casualizado, em esquema de fatorial 2×5×5, com quatro repetições de 50 sementes por tratamento. As variáveis analisadas foram porcentagem de germinação, primeira contagem de germinação, índice de velocidade de germinação, comprimento da parte aérea e da raiz, massa seca total da plântula e extravasamento de eletrólitos das folhas e da raiz. O condicionamento das sementes com ácido ascórbico nas concentrações de 0,50 mM para o genótipo BRS Marataoã e 0,75 mM para o Setentão, possibilitou o desenvolvimento de plântulas mais vigorosas e a redução dos danos às membranas ocasionado pelo estresse oxidativo tanto na ausência do déficit hídrico quanto nos potenciais osmóticos testados, inclusive no mais baixo.

Palavras-chave: *Vigna unguiculata* L.. Estresse oxidativo. Seca.

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INTRODUCTION

Water is one of the most compromising factors in the germination and initial growth of seedlings, as it makes up the matrix necessary for most biochemical and physiological processes (TAIZ *et al*., 2017). When water is available, tissues are hydrated and respiration is intensified (CARVALHO; NAKAGAWA, 2012; MARCOS FILHO, 2015), and control is maintained over the structure and property of proteins, membranes, nucleic acids and other cellular constituents (TAIZ *et al*., 2017).

Water restrictions in the soil, a common phenomenon in arid and semi-arid regions, cause a reduction in the water potential of plants and, consequently, in turgor, increasing ionic toxicity and inhibiting photosynthesis, leading to a limitation on the physiological and biochemical functions of the cells. Faced with these conditions, plants activate complex mechanisms of response to water scarcity, involving detrimental and/or adaptive changes, to avoid or tolerate the period of stress (LISAR *et al*., 2012).

After sowing, periods of prolonged water deficit can cause seeds to deteriorate, impairing their physiological potential, which begins to interfere with seedling emergence, causing a reduction in the number of plants in the field (CARVALHO *et al*., 2016). Furthermore, a lack of water in the soil often causes oxidative stress in plants (BARBOSA *et al*., 2014), and results in the formation of reactive oxygen species (ROS) in the cells, particularly the superoxide anion (O_2^-), the hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2) (MAIA *et al*., 2012). Normally, the generation and elimination of ROS is in a state of dynamic equilibrium, but when under stress, there is an imbalance in this relationship, which results in the accumulation of ROS (KRANNER *et al*., 2010).

An excess of reactive oxygen species causes damage to membranes and macromolecules, affects the cellular metabolism of plants under water stress, and can lead to cell death (BARBOSA *et al*., 2014; LISAR *et al*., 2012). Among the main consequences of ROS accumulating in the cell are lipid peroxidation, protein oxidation, enzymatic inhibition, DNA and RNA damage and, consequently, senescence, poor photosynthesis and leaf necrosis (SCANDALIOS, 2005).

Under such conditions, plants try to maintain their water potential by accumulating osmoprotectants and/or compatible solutes inside the cell, regardless of the volume resulting from the loss of water (TAIZ *et al*., 2017), in addition to adopting structural resources to improve cell function under water stress (LISAR *et al*., 2012). Ascorbic acid is considered a cellular molecule with great antioxidant potential, thanks to its chemical properties of donating electrons to a large number of enzyme and non-enzyme reactions (GILL; TUTEJA, 2010), protecting organelles and cells against ROS, and preventing their stress-induced accumulation (BELTAGI, 2008).

Because it is soluble in water, ascorbic acid is advantageous as a conditioning agent, where its exogenous application is indicated as a way of combatting stress in seeds due to its action in inducing stress-tolerant proteins (MCCUE *et al*., 2000), and protecting against lipid peroxidation and reactive oxygen species induced by ageing (FOYER; NOCTOR, 2005). Ascorbic acid also promotes an improvement in germination potential in oilseeds when subjected to prolonged storage (DOLATABADIAN; MODARRES SANAVY, 2008) or in artificially aged seeds (KRANNER *et al*., 2006). Various studies have been developed to evaluate the benefits of AsA in treating seeds for protection against biotic and abiotic stress. When subjected to water restriction, simulated by increasing levels of salt, an increase in germination and an improvement in the development of seedlings in the field has been reported in crops such as potatoes (VENKATESH *et al*., 2012), peas (ALQURAINY, 2007), beans (ALQURAINY, 2007), sorghum (ARAFA; KHAFAGY; EL-BANNA, 2007) and tomatoes (ZHANG *et al*., 2011).

Given the above, the aim of this work was to study the effects of ascorbic acid in conditioning cowpea seeds subjected to water stress.

MATERIAL AND METHODS

The experiment was conducted at the Seed Analysis Laboratory of the Department of Plant Science at the Centre for Agricultural Sciences of the Federal University of Ceará, Pici Campus, Fortaleza, using cowpea seeds of the BRS Marataoã and Setentão genotypes.

First, the degree of moisture in the seeds and the thousand-seed weight were determined as described in Brasil (2009).

The seeds were disinfected by immersing them in a 70% alcohol solution (v/v) and agitating for 30 seconds. They were then quickly washed twice with distilled water and immediately immersed in a 2.5% sodium hypochlorite solution (v/v) for 2 minutes, washed with distilled water and dried on paper towels. The seeds were conditioned on rolls of Germitest® paper, moistened with ascorbic acid solution at a concentration of 0.0, 0.25, 0.50, 0.75 and 1.0 mM in the proportion of 2.5 times the weight of the substrate, for 4 hours at 25 ºC (BRILHANTE *et al*., 2013).

The conditioned seeds were then distributed on Germitest® paper rolls moistened with saline solution (diluted NaCl), in the proportion of 2.5 times the weight of the substrate. The solutions were adjusted to a concentration of 0.0 (control), -0.3, -0.6, -0.9 and -1.2 MPa by diluting the mannitol in distilled water, based on the formula proposed by Van't Hoff, i.e. $\Psi os = -RTC$, where: Ψos = osmotic potential (atm); R = universal ideal gas constant (8.32 J mol^{-1} K^{-1}); T = temperature (K); C = concentration (mol L^{-1}) and T(K) = 273 + T (ºC). Two hundred seeds were used for each treatment, which were distributed on four paper rolls, each of 50 seeds, and placed in a Biochemical Oxygen Demand (BOD) germination chamber, at 25 ºC (BRASIL, 2009).

The first germination count and final germination count were carried out on the fifth and eighth day respectively after setting up the test. The percentage of normal seedlings was considered, using as classification criteria the definitions established in the Rules for Seed Analysis (BRASIL, 2009). The number of normal seedlings was counted daily to determine the germination speed index proposed by Maguire (1962).

Growth of the shoots and main roots was determined eight days after setting up the germination test; ten normal seedlings were selected per treatment and measured with the aid of a rule graduated in centimetres. The seedlings were then placed in a forced air circulation oven at a constant temperature of 65 °C for 72 hours. They were immediately weighed to three decimal places on a precision balance to obtain the dry weight, with the results expressed in milligrams per seedling.

Membrane permeability was determined by electrolyte leakage, on the eighth day after setting up the germination test. Leaf discs and root segments from the middle and upper third of the root system were used, placing approximately 0.1 grams in test tubes containing 10 mL of distilled water. These were then closed and kept for 24 hours at room temperature (25 ºC), after which the initial conductivity (C1) was read using a properly calibrated benchtop conductivity meter. The tubes were subjected to a temperature of 80 ºC for 60 minutes in a water bath and, after their contents had cooled, the final conductivity was taken (C2). Relative permeability was calculated from the equation [C1/(C1+C2)] x 100 (TARHANEN *et al*., 1999).

The experiment was conducted in a completely randomised design, in a triple factorial scheme of 2 genotypes x 5 concentrations of ascorbic acid x 5 osmotic potentials, with four replications. The data were submitted to analysis of variance followed by regression analysis at a significance level of 5% using the SISVAR® statistical software (FERREIRA, 2000). The Sigmaplot v12.5 software was used to display the results graphically.

RESULTS AND DISCUSSION

The seeds showed different values for the degree of moisture and thousand-seed weight, where 13% and 182.5 g was found for the BRS Marataoã genotype, and 11% and 178.3 g for the Setentão genotype respectively.

Percentage germination in the cowpea seeds decreased as the osmotic potential of the substrate was reduced. In the absence of a water deficit, germination was 87% and 97% for the BRS Marataoã and Setentão cultivars respectively; when subjected to -1.2 Mpa, the values decreased to 44% and 14% respectively, with the latter being more sensitive to water stress (Figures 1a and 1b). This reduction can be attributed to the speed and amount of water absorbed by the seeds, since a water deficit inhibits water absorption by the tissues, making it difficult for germination to start (KAPPES *et al*., 2010).

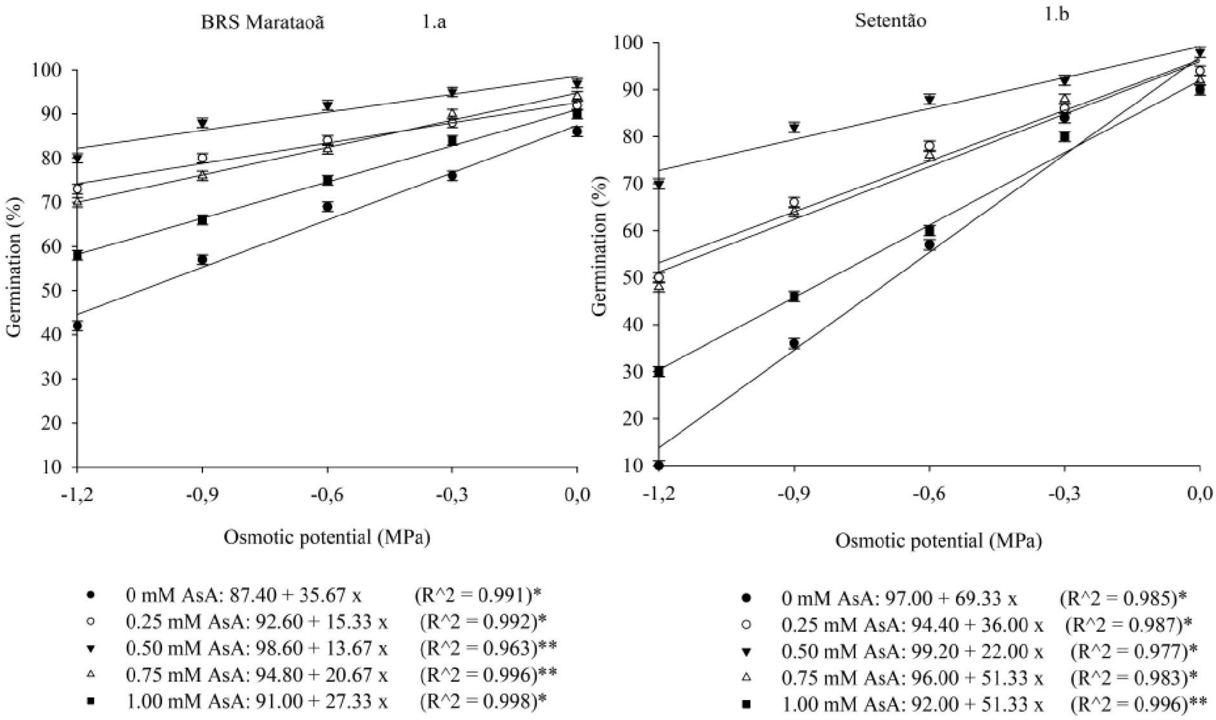
These results agree with those obtained by Viçosi *et al*. (2017), who reported a linear reduction in the germination of bean seeds when exposed to conditions of water stress simulated by mannitol. Duarte *et al*. (2013) also found the same reduction in the same species from an osmotic potential of -0.6 MPa on. However, Custódio, Salomão and Machado Neto (2009), evaluating different osmotic agents, note that a potential of -0.55 MPa simulated by $CaCl_2$ allowed maximum germination in bean seeds, while mannitol and $MgCl_2$ afforded no differences in germination, with values of around 98%. Gomes, Almeida and Takahashi (2015), also found no negative response to germination in the string bean as the osmotic potential gradient was reduced.

Germination in the soya bean was also affected by water stress, as reported by Carvalho *et al*. (2016) when they observed a decrease in this variable for the BRS Tordilha RR, FPS Solimões RR and FPS Paranapanema RR cultivars relative to an increase in water deficit simulated by mannitol, with the first cultivar presenting a reduction of 16% when exposed to -0.84 MPa compared to the control (0.0 MPa).

Treating the cowpea seeds with ascorbic acid (AsA) resulted in an increase in the germination rate of the seeds of the genotypes under evaluation, achieving values of 78% and 68% for BRS Marataoã and Setentão respectively, even when subjected to the lowest potential (-1.2 MPa). This increase is related to an increase in gibberellin biosynthesis, a result of the exogenous application of AsA from the conditioning process (KHAN; MAZID; MOHAMMAD, 2011).

The genotypes showed similar behaviour in response to the application of ascorbic acid in the face of water stress, where a concentration of 0.50 mM AsA gave the highest values, with an average germination of 90% up

Figura 1 - Germination of cowpea seeds at different osmotic potentials and conditioned with ascorbic acid



* significant at 5%; ** significant at 1%

to -0.6 MPa, reducing as the potential decreased (Figures 1a and 1b).

Ascorbic acid is a natural antioxidant that plays an important role in plant metabolism as it is involved in embryogenesis during the process of seed formation (RAZA *et al*., 2013). Its exogenous application may allow an endogenous increase in this antioxidant; but when applied at high concentrations, AsA can become detrimental, by nullifying (TAKEMURA *et al*., 2010) or simply reducing germination, as reported by Ishibashi and Iwaya-Inoue (2006) in wheat seeds treated with 50 and 100 mM ascorbic acid.

Conditioning seeds with AsA to promote germination in the face of a reduction in the osmotic potential of the substrate has been reported by some authors, including Dehghan, Rezazadeh and Habibi (2011), who applied 400 ml L^{-1} AsA to seeds of the soya bean, which resulted in an increase in percentage germination, even at 50 mM NaCl (approximately -0.15 MPa).

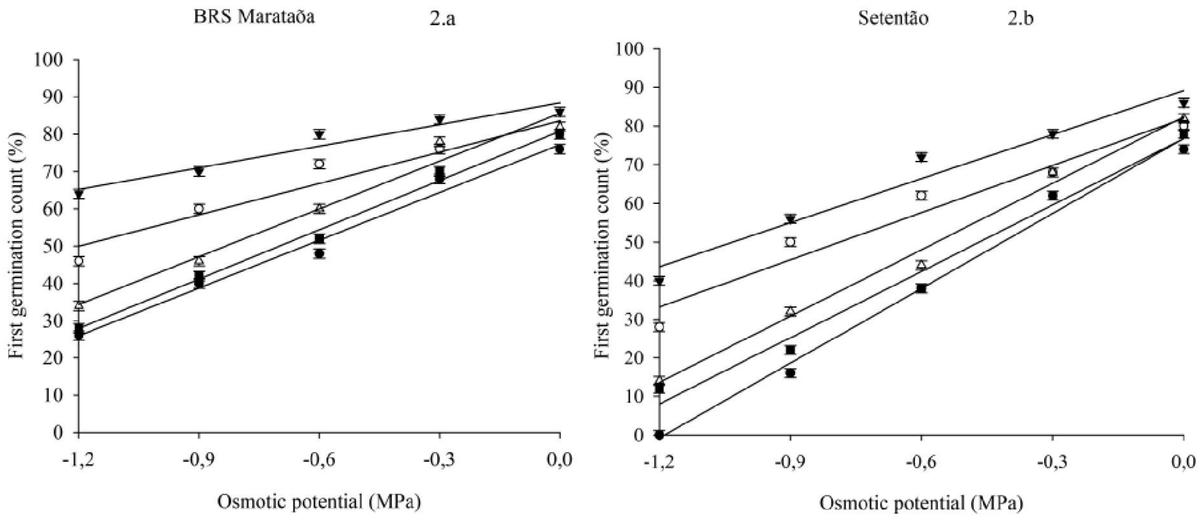
Just as in the germination test, the first germination count was influenced by the AsA concentration and the water deficit. Once again, it can be seen that treating the seeds with ascorbic acid was effective in resisting the damage caused by water scarcity, favouring a concentration of 0.50 mM for the BRS Marataoã and Setentão genotypes (Figures 2a and 2b).

Based on the values seen for first germination count, water deficiency affected the germination speed of the cowpea genotypes under evaluation. Deuner *et al*. (2011) found the same behaviour in cowpea genotypes, reporting a delay and reduction in germination from a concentration of 50 mM NaCl on (approximately -0.15 MPa). Soya beans were also affected by water deficit, with the seeds not even germinating when exposed to -0.9 Mpa.

For the germination speed index, greater speed was seen in the control treatment (0.0 MPa). Furthermore, as the osmotic potential of the substrate decreased, the seeds needed more time for soaking and to germinate (Figures 3a and 3b).

Seeds conditioned with AsA, were able to soak and germinate more quickly. In the BRS Marataoã genotype, the germination speed index had a value of 13 both in unconditioned seeds and in the control treatment (0.0 MPa). When conditioned, these values increased, reaching 16 seedlings at a concentration of 1.00 mM

Figura 2 - First germination count of cowpea seeds at different osmotic potentials and conditioned with ascorbic acid

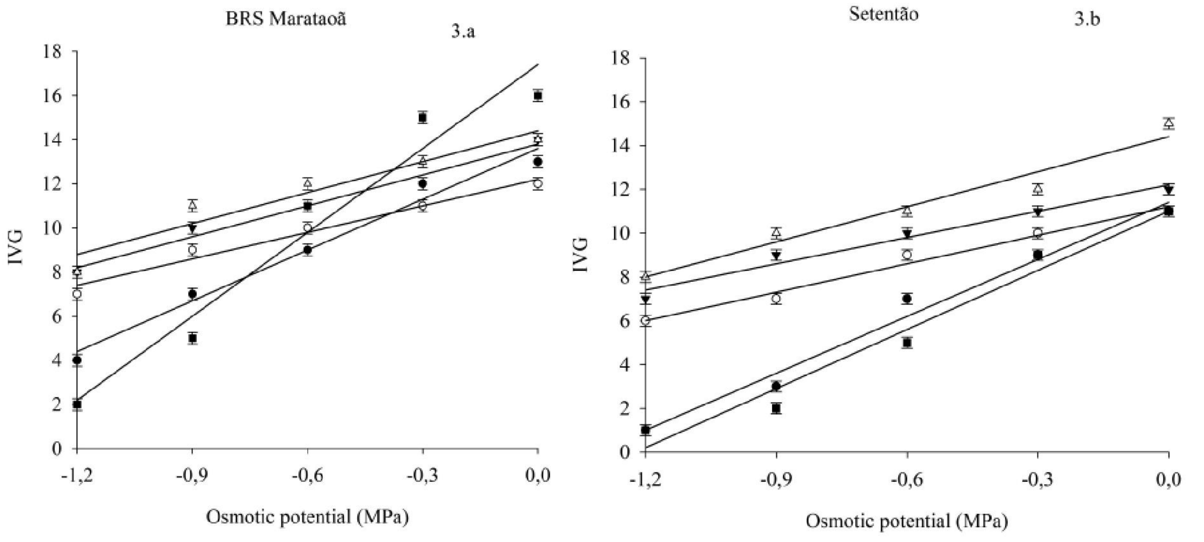


- 0 mM AsA: 77.20 + 42.67 x (R^2 = 0.991)**
- 0.25 mM AsA: 83.60 + 28.00 x (R^2 = 0.961)**
- 0.50 mM AsA: 88.40 + 19.33 x (R^2 = 0.971)**
- 0.75 mM AsA: 85.60 + 42.67 x (R^2 = 0.988)**
- 1.00 mM AsA: 80.80 + 44.00 x (R^2 = 0.996)**

- 0 mM AsA: 76.80 + 64.67 x (R^2 = 0.995)**
- 0.25 mM AsA: 82.00 + 40.67 x (R^2 = 0.976)**
- 0.50 mM AsA: 89.20 + 38.00 x (R^2 = 0.979)**
- 0.75 mM AsA: 82.40 + 57.33 x (R^2 = 0.996)**
- 1.00 mM AsA: 76.80 + 57.33 x (R^2 = 0.991)**

* significant at 5%; ** significant at 1%

Figura 3 - Germination speed index of cowpea in different osmotic potentials and conditioned with ascorbic acid



- 0 mM AsA: 13.60 + 7.67 x (R^2 = 0.990)*
- 0.25 mM AsA: 12.20 + 4.00 x (R^2 = 0.986)*
- 0.50 mM AsA: 13.80 + 4.67 x (R^2 = 0.990)*
- 0.75 mM AsA: 14.40 + 4.67 x (R^2 = 0.962)**
- 1.00 mM AsA: 17.40 + 12.67 x (R^2 = 0.979)*

- 0 mM AsA: 11.40 + 8.67 x (R^2 = 0.991)*
- 0.25 mM AsA: 11.20 + 4.33 x (R^2 = 0.991)**
- 0.50 mM AsA: 12.20 + 4.00 x (R^2 = 0.986)*
- 0.75 mM AsA: 14.40 + 5.33 x (R^2 = 0.977)**
- 1.00 mM AsA: 11.00 + 9.00 x (R^2 = 0.985)*

* significant at 5%; ** significant at 1%

ascorbic acid. When treated with 0.75 mM AsA and subjected to the lowest osmotic potential (-1.2 MPa), the GSI reached a value of 8, a similar value to that obtained in untreated seeds exposed to -0.6 MPa (Figure 3a). In the Setentão genotype, the concentration of 0.75 mM AsA gave the highest GSI values Figure 3b). The results can be explained by the ascorbic acid affecting several physiological processes, including metabolic changes in plants under conditions of water deficit to favour the availability of water and nutrients (KHAN; MAZID; MOHAMMA, 2011).

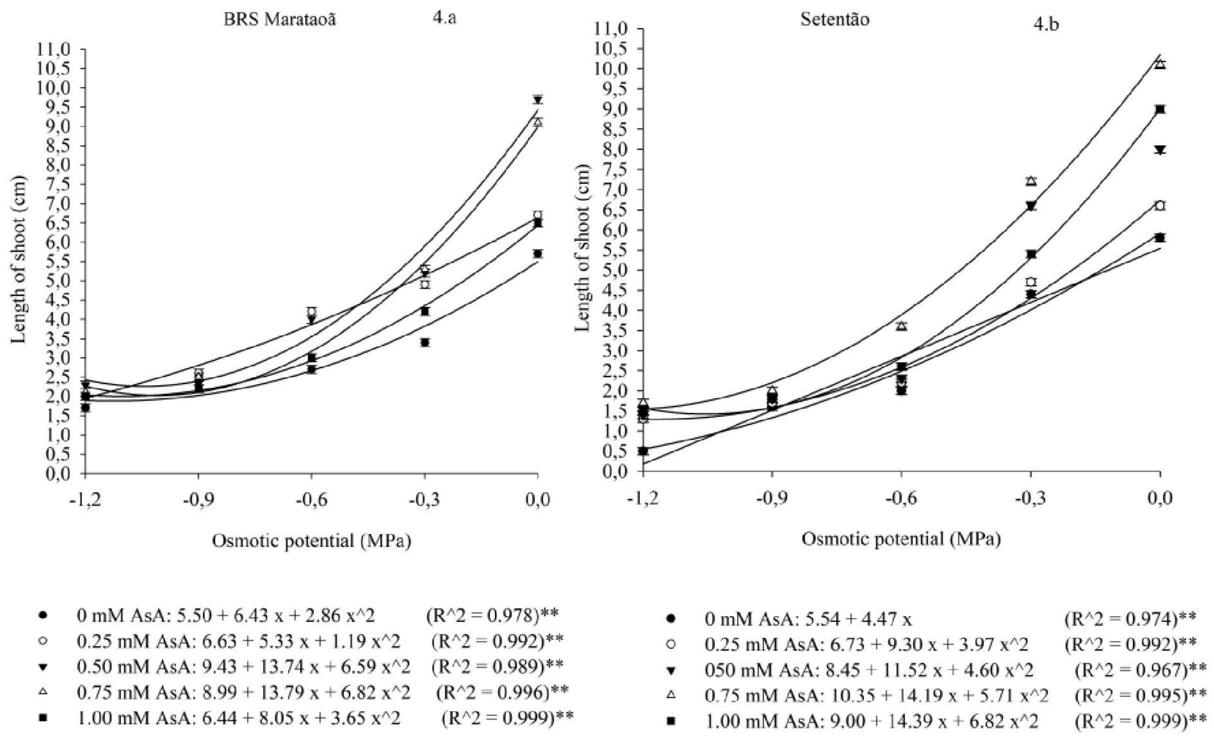
When evaluating seedling growth, it was found that the cowpea genotypes were highly sensitive to water deficit, which directly affected shoot development (Figure 4).

Analysing shoot growth by adjusting the regression curves for the genotypes under evaluation, it was found that they showed a similar trend in response to the treatments being used. For seedlings that were not treated with ascorbic acid, but subjected to different water potentials, there was a constant fall in this variable, reaching values of 1.7 and 0.5 cm for BRS Marataoã and Setentão respectively at -1.2 MPa. Water stress promotes a reduction in the osmotic potential of the cell, and consequently, a reduction in turgor pressure, hampering the expansion and growth of the cell, thereby reducing plant growth (JALEEL *et al*., 2009).

Conditioning seeds with AsA at a concentration of 0.50 and 0.75 mM resulted in greater seedling growth, reaching mean values of 2.3 and 1.7 cm for the BRS Marataoã and Setentão genotypes respectively when exposed to the lowest potential under test. Even when not subjected to water stress, the treated seeds proved to be more vigorous compared to the untreated seeds, which can be seen from the length of the shoots, where in the BRS Marataoã genotype the length was was 9.7 cm, while in the untreated plant it was 5.7 cm (Figure 4a); for the Setentão genotype, this value was 10.1 and 5.8 cm for the treated and untreated plants respectively (Figure 4b).

Studies which aimed to evaluate the effects of water deficit on initial seedling growth have been carried out, among them, that of Gomes, Almeida and Takahashi (2015), who report that the shoots of string bean seedlings showed no difference in initial growth at potentials of 0.0 to -1.2 MPa, but noting a reduction in this variable at lower potentials. Viçosi *et al.* (2017) also reported a reduction in the growth of the hypocotyl

Figura 4 - Shoot length of cowpea seedlings at different osmotic potentials and conditioned with ascorbic acid



* significant at 5%; ** significant at 1%

in bean plants: for the control treatment (0.0 MPa), they found an increase of 11 mm, while at -2.4 MPa, the increase was only 3 mm.

When treating seeds of the DPX cultivar of the soya bean with 400 mg L^{-1} AsA, Dehghan, Rezazadeh and Habibi (2011) found that seedlings induced to an osmotic stress of 50 mM NaCl (approximately -0.1 MPa) presented a mean length greater than that obtained in the control treatment (no salt and untreated seeds). In pea plants, El-Hak, Ahmed and Moustafa (2012), found that the application of 200 ppm ascorbic acid to the leaves resulted in larger and more vigorous plants.

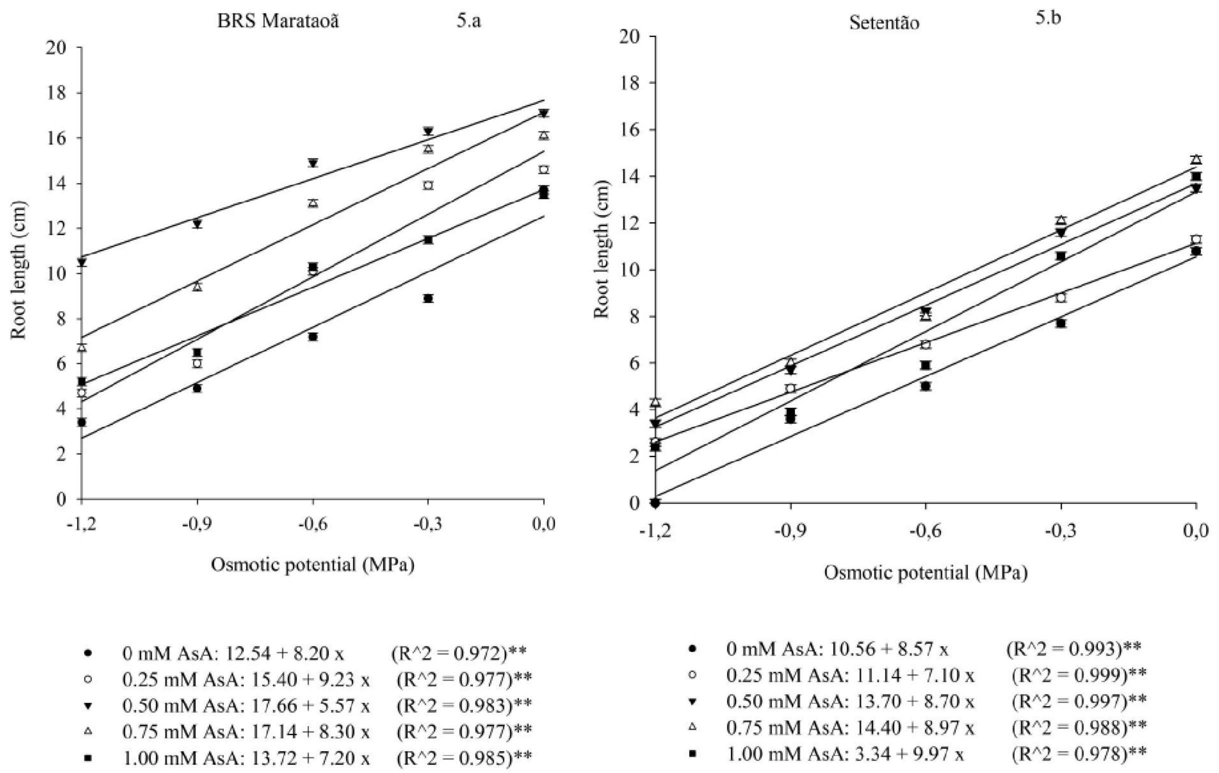
Root growth in the cowpea seedlings was affected by the factors under analysis. For the BRS Marataoã genotype, the greatest values occurred in seeds treated with 0.50 mM AsA, where, even at the lowest osmotic potential (-1.2 MPa), this value was greater than the value obtained in the control treatment when exposed to -0.3 MPa. The same can be seen for the Setentão genotype in seeds treated with 0.75 mM ascorbic acid. In both genotypes, the shortest root length was seen in the absence of AsA (Figures 5a and 5b).

These results agree with those found by other authors, among them, Custódio, Salomão and Machado Neto (2009), who saw a reduction in the root growth of bean seeds as the water deficiency increased. Deuner *et al*. (2011), also reported a reduction in this variable relative to the control in cowpea genotypes subjected to an osmotic potential of 200 mM NaCl (approximately -0.5 MPa). The root system is extremely important for absorbing water and nutrients and supporting the plant. Problems in the formation of its structure can hamper its establishment and so impair crop yield (CARVALHO *et al*., 2016).

There was a reduction in the dry matter of the cowpea seedlings for the genotypes under evaluation as the osmotic potential was reduced (Figure 6).

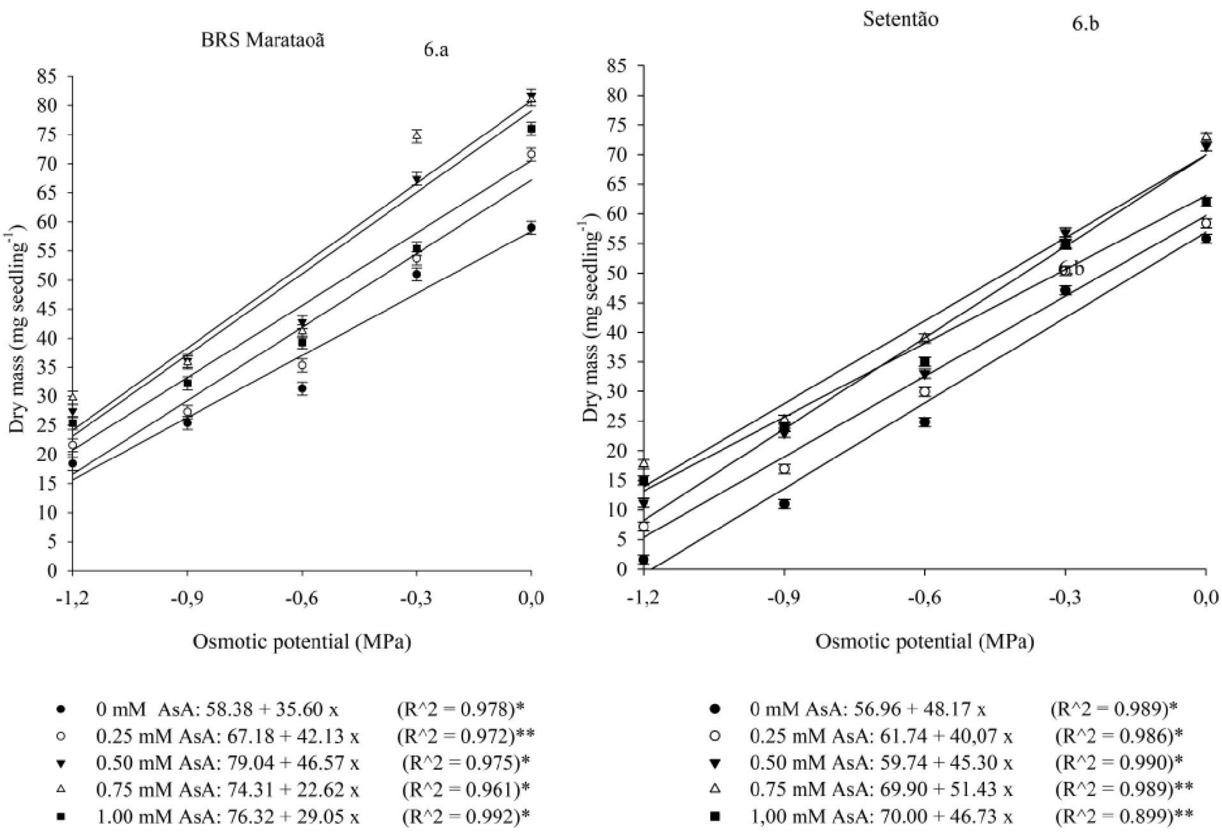
Studies have reported a reduction in seedling biomass in the face of water stress. Custódio, Salomão and Custódio Neto (2009), noted that both shoot and root dry matter decreased as the water deficiency increased during germination. However, Deuner *et al*. (2011) stated that in plants of *Vigna radiata*, doses of up to 150 mM NaCl (approximately -0.4 MPa) resulted in an

Figura 5 - Root length of cowpea seedlings at different osmotic potentials and conditioned with ascorbic acid



* significant at 5%; ** significant at 1%

Figura 6 - Dry mass of cowpea seedlings at different osmotic potentials and conditioned with ascorbic acid



* significant at 5%; ** significant at 1%

increase in stem dry matter, while the same was found for the leaves up to a dose of 100 mM (approximately -0.35 MPa).

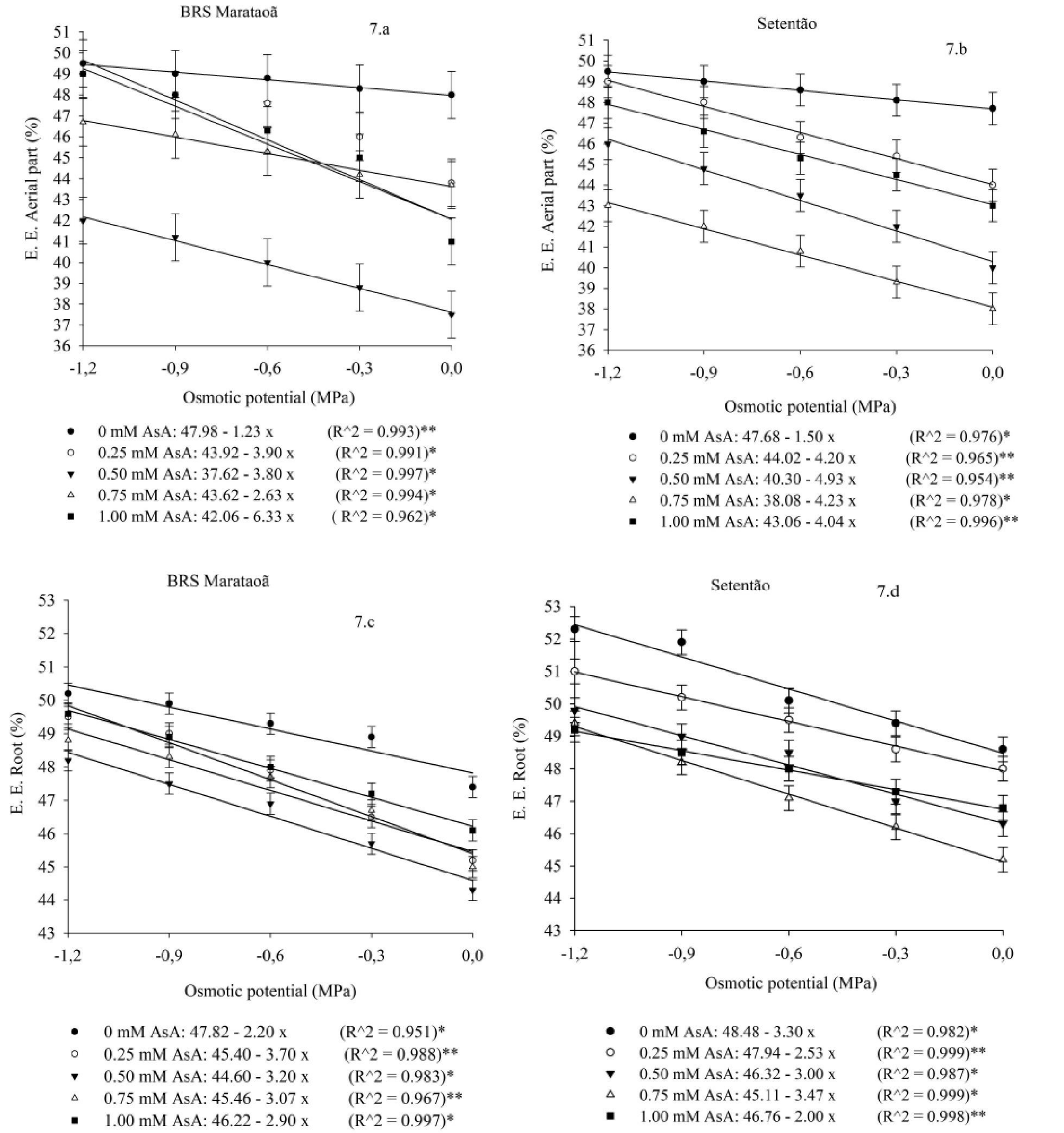
The water deficit caused increased damage to the membranes; this was due to electrolyte leakage in the leaf and root tissue of the BRS Marataoã and Setentão genotypes. At the lowest osmotic potential, the release of electrolytes in these tissues increased by 1.1 times compared to the respective controls (Figures 7a and 7b), with the root system releasing more electrolytes than the leaves (Figures 7 and 7d). Maia *et al*. (2012) also found a steady increase in the release of electrolytes in cowpea cultivars in relation to the control treatment when the seeds were subjected to an osmotic potential of 100 mM NaCl (approximately -0.25 MPa).

Conditioning with ascorbic acid enabled an increase in the defence mechanism of the cowpea seedlings against drought. For seeds of the BRS Marataoã genotype at a concentration of 0.50 mM AsA, membrane damage at the lowest osmotic potential (-1.2 MPa) was less than or similar to that of the control treatment (untreated and no water deficit): 42.00% and 48.00%, and 48.20% and 47.50%, for the leaves and root system respectively.

The other concentrations of AsA also afforded reductions in electrolyte leakage (Figures 7a and 7c). For the Setentão genotype, lower values were obtained for all the AsA concentrations under test than those found in the untreated seeds. For the control treatment, values of 47.7% and 48.6% were found; when the seeds were treated with 0.75 mM AsA and exposed to -1.2 MPa, a reduction in these values was noted, to around 43.00% and 49.40% for the leaves and roots respectively (Figures 7b and 7d).

Ascorbic acid is considered an antioxidant of great importance to plants, as it is essential for the synthesis of hormones and a series of antioxidant enzymes (KHAN; MAZID; MOHAMMAD, 2011), protecting macromolecules from oxidative damage caused by reactive oxygen species (DEHGHAN; REZAZADEH; HABIBI, 2011).

Figura 7 - Extravasation of electrolytes in leaf and root tissues of cowpea genotypes at different osmotic potentials and conditioned with ascorbic acid



* significant at 5%; ** significant at 1%

CONCLUSION

Conditioning the seeds with ascorbic acid at a concentrations of 0.50 mM for the BRS Marataoã genotype and 0.75 mM for the Setentão genotype, enabled the development of more vigorous seedlings and a reduction in the damage caused to the membranes by oxidative stress, both in the absence of a water deficit and at the osmotic potentials under test, including at the lowest potential.

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