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Priming cycles with elicitors of salt stress tolerance in seeds of the cowpea[1]

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ABSTRACT - Exposing seeds to agents that elicit tolerance to abiotic stress, such as phytohormones and organic acids, during hydration and dehydration cycles can determine their response to later stimuli, e.g. exposure to salt stress. The aim of this study was to evaluate the action of priming cycles with different eliciting agents of salt stress tolerance on seeds of the cowpea varieties Sempre Verde and Pingo de Ouro. The seeds were subjected to the following treatments: 0.0 mM NaCl (control); 100 mM NaCl (salt stress); salt stress + three seed-priming cycles (PC) in water; salt stress + PC in gibberellic acid; salt stress + PC in hydrogen peroxide; salt stress + PC in salicylic acid; salt stress + PC in ascorbic acid. The following variables were analysed: germination, growth, dry weight, salt tolerance index, total soluble sugars, total free amino acids and proline. Salt stress (100 mM NaCl) reduced germination, length and biomass accumulation in the Sempre Verde and Pingo de Ouro varieties. These showed the best response to the priming cycles with gibberellic and salicylic acids, which promoted greater germination potential, length and biomass under a salt stress of 100 mM NaCl, affording greater tolerance via osmotic regulation, especially in the Sempre Verde variety.

Key words: *Vigna unguiculata*. Abiotic stress. Seed hydration memory. Attenuators.

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INTRODUCTION

Salinisation is a global problem that has led to the loss of land resources, with a negative impact on the environment, on agricultural production and on socio-economic and cultural factors, as well as on human health. The problem may be aggravated in arid and semi-arid regions due to the rate of evaporation being higher than that of precipitation, favouring the formation of salt crusts in the surface layers of the soil (Hassani; Azapagic; Shokri, 2021).

The cowpea (*Vigna unguiculata* L. Walp.) shows sensitivity to salt stress starting with germination and early seedling development (Praxedes *et al*., 2020; Sá *et al*., 2016). Excess ions, especially Na^+ and/or Cl^-, reduce uniform germination, and their presence in the root zone makes it difficult for seedlings to become established in the field by reducing the ability of the plants to extract water from the substrate (Pereira *et al*., 2023; Praxedes *et al*., 2020).

To reduce osmotic potential in the cells, and maintain vital metabolic activities and cell turgidity, cowpea seeds and seedlings produce compatible osmolytes to improve tolerance to abiotic stress (Santos *et al*., 2022). Salt tolerance can therefore be enhanced by adopting pre-germination techniques, such as hydropriming, which results in improved germination potential and uniformity, and the formation of more vigorous cowpea seedlings when these are produced under salt stress (Nabi *et al*., 2020).

When hydropriming is carried out in cycles, it meets the need for the water that the seeds require to activate their metabolism. The start of seed hydration activates the metabolic functions of energy and respiration and the start of reserve mobilisation. Research shows that such pre-germination treatment can be effective for the species, increasing tolerance to abiotic stress (Gonçalves *et al*., 2020; Nascimento; Dantas; Meiado, 2021).

The adoption of pre-germination treatment using other agents that elicit tolerance to abiotic stress, such as plant hormones and organic acids, has been reported in the literature during the initial stages of plant development, with the aim of improving germination, emergence and seedling formation in rice (*Orysa sativa*) (Garcia *et al*., 2021), sorghum (*Sorghum bicolor* (L.) Moench) (Ali *et al*., 2021) and maize (*Zea mays* L.) (Pereira *et al*., 2023).

In rice seedlings, gibberellic acid improves water absorption, increases cell membrane plasticity, stimulates amylase activity and allows insoluble starch to be converted into soluble sugars (Garcia *et al*., 2021; Miri *et al*., 2021). The exogenous application of gibberellic acid improves plant survival under salt stress (Sá *et al*., 2020). In the cowpea, priming with ascorbic acid helps activate the defence mechanism of the seedlings, protecting them against oxidative damage caused by excess salts in the plant cells (Nunes *et al*., 2019). In turn, salicylic acid is a phytohormone that is well known as a signalling molecule, inducing the antioxidant defence system of the plants to biotic or abiotic stress and which, when absorbed by the seeds, acts as an indicator of stress (Chen; Cao; Niu, 2021).

The aim of this study, therefore, was to assess, using physiological and biochemical evaluations, the action of priming cycles with different eliciting agents of salt stress tolerance on seeds of the cowpea.

MATERIAL AND METHODS

The experiment was conducted at the Seed Analysis Laboratory of the Department of Agricultural and Forestry Sciences at the Federal Rural University of the Semi-arid Region (UFERSA), Mossoró, Rio Grande do Norte, Brazil (5° 11’ S, 37° 20’ W and altitude of 18 m).

The experiment was conducted in a completely randomised 2 x 7 factorial design, with four replications of 50 seeds. The treatments consisted of two cowpea landrace varieties (Sempre Verde and Pingo de Ouro) and seven combinations of priming cycles (PC) with elicitors of salt stress tolerance: T1 – 0.0 mM NaCl (control) with no PC; T2 – 100 mM NaCl (salt stress); T3 – three seed priming cycles (PC) with distilled water + salt stress; T4 – PC with gibberellic acid – 50 µM (GA_3) + salt stress; T5 – PC with hydrogen peroxide – 5 mM (H_2O_2) + salt stress; T6 – PC with salicylic acid – 50 µM (SA) + salt stress, and T7 - PC with ascorbic acid – 50 µM (ASC) + salt stress (Table 1).

The cowpea varieties used in the experiment, Sempre Verde and Pingo de Ouro, come from the collections of Landrace Seed Guardians in rural communities in the district of Caraúbas, Rio Grande do Norte, have an indeterminate growth habit and were harvested in 2019. Once received, the seeds were stored in a controlled environment (16 ºC-18 ºC and relative humidity of 40%) throughout the experimental phase. The Sempre Verde variety has light greenish-brown grains and is widely grown in the northeast of Brazil due to its early cycle of 68 days and average yields of 1,022 kg ha^{-1} and 1,782 kg ha^{-1} under rainfed and irrigated regimes, respectively. Pingo de Ouro is a semi-early type and is widely grown in the northeast of the country, with a cycle of up to 80 days and an average green-grain production of 636.9 kg ha^{-1}.

Initially, the water content of the seeds was quantified using the oven method at 105 ± 3 °C for 24 hours (Brasil, 2009), in two repetitions of 4.5 ± 0.5 g. The water content was calculated on a wet basis and expressed as a percentage.

The imbibition curve was determined in two replications of 50 seeds, which were initially weighed on a digital analytical balance (0.001 g), then again after each previously determined time interval until emission of the primary root. Imbibition was carried out using the water immersion method with the seeds placed in a beaker containing 100 mL of distilled water at 25 ºC. Weighing took place every hour during the first eight hours of soaking, then every two hours until thirty-two hours had elapsed, at which point the primary root had protruded in 50% of the seeds in each replication (Figure 1).

While soaking the seeds, the weight gain was calculated as per the formula proposed by Cromarty, Ellis and Roberts (1985): weight gain (%) = [(Wf – Wi)/Wi] x 100, where Wf: final weight (gain in moisture for each period of soaking) and Pi: initial weight of the seeds before soaking.

Figure 1- Imbibition curve in seeds of the cowpea varieties Sempre Verde and Pingo de Ouro, using the water immersion method at 25°C. Start of root protrusion (RP)

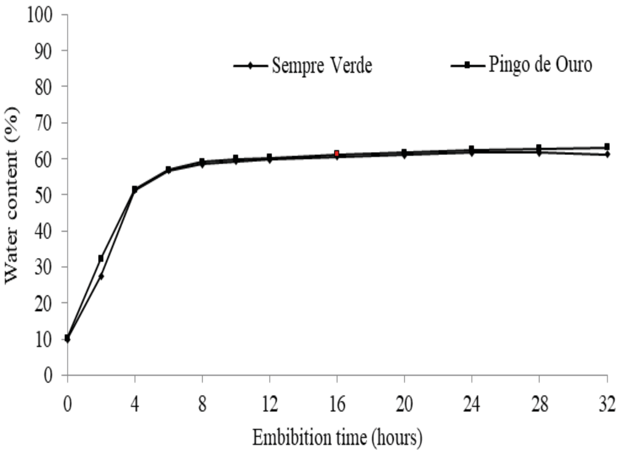


Table 1 - Electrical conductivity (EC) of the solutions used in the priming cycles of seeds of the cowpea varieties Sempre Verde and Pingo de Ouro

Solutions	EC (μSm^{-1}) at 25 ºC
Distilled water	4.13
50 µM gibberellic acid	14.25
5 mM hydrogen peroxide	4.17
50 µM salicylic acid	14.96
50 µM ascorbic acid	9.40

The priming cycles were defined based on the data obtained from the imbibition curve, while the concentrations of both salt and the tolerance eliciting agents were determined from preliminary tests and by following the guidelines of Pereira *et al*. (2023) for maize. The hydration process was carried out in 50 mL of elicitor per cycle in disposable cups that contained 100 seeds and were placed for thirty minutes in the dark in a germination chamber at 25 °C. The seeds were then dried and placed on paper towels, where they were left to dehydrate for twelve hours in three cycles. Drying was carried out at an average ambient temperature of 29.5 ± 0.7 °C and relative humidity of 67 ± 5%, determined using a Jprolab® digital thermo-hygrometer. At the end of the priming cycles, the water content of the seeds was around 30%.

Following the priming cycles, the seeds were sown on paper-roll substrate previously moistened with distilled water (0.0 mM) for the control, and with salt water (100 mM), obtained by dissolving 5.84 g sodium chloride (NaCl) L^{-1}, for the other treatments. The rolls were incubated in a germinator at 25 °C (Brasil, 2009). First germination count (FGC) and germination (G) were assessed five and eight days after sowing, respectively (Brasil, 2009).

The shoot (SL) and root (CR) length of normal seedlings were measured at the end of the germination test. The length of the shoots (from the collar to the apex of the seedling) and of the primary root (from the base of the collar to the end of the root) were measured using a ruler graduated in centimetres. The seedlings were then placed in kraft paper bags and left to dry in a forced air circulation oven at 65 °C to constant weight. They were then weighed on a precision balance to obtain the dry weight of the cotyledons (COTDW), shoots (SDW) and roots (RDW).

From the results of the shoot and root dry weight (DW), the percentages for the vegetative organs, and the salt tolerance index of the shoots and roots were calculated, comparing the data from the salt treatments with those of the control (EC = 4.13 μSm^{-1} at 25 ºC). The tolerance was classified into four levels based on the loss of biomass: T (tolerant; 0 – 20%), MT (moderately tolerant; 21 – 40%), MS (moderately sensitive; 41 – 60%), and S (sensitive; >60%), using the equation adapted from Fágeria, Soares and Gheyi (2010):

$$TI(\%)=\frac{DW\ \ from\ the\ salt\ treatment}{DW\ \ from\ the\ control\ treatment}\times 100 \qquad (1)$$

Total soluble sugars (TSS) were determined from the fresh weight of the seedlings. For extraction, the material was macerated in liquid nitrogen using a pestle and mortar. Then, 0.2 g was weighed in triplicate and placed in Eppendorf screw-cap tubes. One mL of 80% alcohol was added and the samples were placed in a water bath at 60 ºC for 20 min. The material was centrifuged at 4 °C for 10 min at 10 rpm (this process was repeated three times) and the supernatant was collected to quantify the total soluble sugars, measured by spectrometry at 620 nm using the anthrone method (Yemm; Willis, 1954), with glucose as the standard and the results expressed as µmol GLU g^{-1} fresh matter.

The supernatant obtained in the extraction process was used to quantify the total free amino acid content (AA) using the acid nihydrin method. The absorbance was measured at 570 nm (Yemm; Cocking; Ricketts, 1955), with glycine as the standard substance and the results expressed in µmol GLY g^{-1} fresh matter.

The concentrations of the amino acid proline (PRO) were determined as per the methodology described by Bates *et al*. (1973), based on the standard curve obtained from L-Proline and measuring the absorbance at 520 nm. The results were expressed in µmol PRO g^{-1} fresh matter.

The data were submitted to analysis of variance (F-test). The mean values of the pre-germination treatments within each variety were compared using the Scott-Knott test at 5% probability, while the mean values of the varieties within each treatment were compared using Student's t-test at 5% probability. The statistical analyses were carried out using the System for Analysis of Variance – SISVAR software (Ferreira, 2019).

RESULTS AND DISCUSSION

The physiological parameters of the cowpea seedlings (Sempre Verde and Pingo de Ouro) decreased significantly under a salt stress of 100 mM NaCl with no priming cycles compared to the control. The interaction between the cowpea varieties and the pre-germination treatments was significant ($p < 0.01$) for first germination count, germination, shoot and root length of the cotyledon dry matter, shoot dry weight and root dry weight (Table 2).

Salt stress with no priming cycles reduced the first germination count in the Sempre Verde and Pingo de Ouro varieties by 56 and 43 percentage points, respectively, compared to the control. The difference in first count and final germination between the two varieties in the control treatment is related to the phenotypic characteristics of each variety, particularly Pingo de Ouro, which resulted in greater germination potential under ideal conditions of temperature, substrate and light (Figure 2).

This difference does not affect the response of the varieties under a salt stress of 100 mM NaCl, with a reduction in the number of normal seedlings germinated at the first count, which continued until final germination. This is similar for the Pingo de Ouro variety under a salt stress of 8.0 dSm^{-1} (Sá *et al.*, 2016). In contrast, Loiola *et al.* (2022) found that a salt stress of 4.5 dSm^{-1} favoured an increase in germination for the two varieties under study, and underline that in the cowpea, higher salinity affects germination.

Priming cycles with tolerance elicitors improved first germination count for the Sempre Verde variety in relation to salt stress with no priming cycles. However, the priming cycles with gibberellic and salicylic acids increased germination by 30 and 27 percentage points, respectively (Figure 2A). For the Pingo de Ouro variety, priming with tolerance elicitors favoured an increase in germinated seedlings at the first germination count, while those with salicylic acid had the best results, by 24 percentage points, compared to salt stress with no priming cycles.

Salt stress with no priming cycles affected the germination potential of the Sempre Verde and Pingo de Ouro varieties by 67 and 73 percentage points, respectively (Figure 2B). Priming cycles with tolerance elicitors

Table 2 - Analysis of variance for the variables first germination count (FGC), germination (G), shoot length (SL), root length (RL), cotyledon dry weight (COTDW), shoot dry weight (SDW) and root dry weight (RDW) in seeds of the cowpea varieties Sempre Verde and Pingo de Ouro subjected to priming cycles with elicitors of salt stress tolerance

SV	DF	Mean square						
		FGC (%)	G (%)	SL (cm)	RL (cm)	COTDW (mg)	SDW (mg)	RDW (mg)
Treatments	6	1803.03**	4535.11**	61.29**	34.96**	4053.87**	692.47ns	171.28**
Cultivars	1	795.02**	1107.16**	21.75**	33.79**	17611.56**	118.90**	9.12*
Treat. x Cult.	6	68.43**	363.49**	2.61**	8.79**	390.13**	156.11**	10.68**
Error	42	13.42	9.73	0.40	0.88	70.68	35.48	1.71
CV (%)		16.35	7.32	11.47	8.13	10.64	14.89	8.29
Mean value		22.4	42.6	5.5	11.5	79.6	40.0	15.8

SV: source of variation; GL: degrees of freedom; **; *: significant effect at 0.01 and 0.05 significance improved germination in relation to salt stress with no priming; however those with salicylic acid resulted in a greater number of germinated seedlings, corresponding to 71% for the Sempre Verde variety and 40% for Pingo de Ouro. The highest values for germination occurred in the Sempre Verde variety, with no statistical difference between varieties for salt stress with no priming cycles and priming cycles with ascorbic acid.

Priming cycles with salicylic acid promoted a significant increase in normal germinated seedlings at the final count in the Sempre Verde and Pingo de Ouro varieties under salt stress with no priming cycles. The action of this phytohormone can be explained by regulation of the plant defence response related to the activation and deactivation of genes linked to gibberellin biosynthesis (crosstalk), which were previously deactivated by the presence of salts and are reactivated by the application of salicylic acid. (Liu *et al*., 2018, 2022; Verma; Ravindran; Kumar, 2016).

Other parameters confirm the effect of salt stress on cowpea varieties, such as a reduction in seedling length in both the shoots and roots, reflected in a reduction in seedling biomass under a salt stress of 100 mM NaCl. Shoot length in seedlings of the Sempre Verde and Pingo de Ouro varieties was reduced by 77.6% and 70.8%, respectively, when the seeds were sown under salt stress with no tolerance elicitors (Figure 3). In the Sempre Verde variety, the priming cycles with tolerance elicitors promoted greater shoot length, especially those with gibberellic acid under salt stress, with gains of 164% in relation to salt stress with no priming cycles (Figure 2C). In the Pingo de Ouro variety, priming cycles with tolerance elicitors also promoted a gain in shoot length, except for the cycles with ascorbic acid, which did not differ from salt stress with no priming cycles (Figure 2C). For this variety, the priming cycles with gibberellic acid promoted greater shoot length (137%), compared to salt stress with no priming cycles, albeit not differing statistically from Sempre Verde (Figure 2C).

Salt stress with no priming cycles reduced RL in the Sempre Verde and Pingo de Ouro varieties by 21% and 31.1%, respectively (Figure 2D). For the Sempre Verde variety, the priming cycles with salicylic acid promoted an increase in RL of 19.2% compared to salt stress with no PC (Figure 2D). For Pingo de Ouro, the priming cycles with salicylic acid increased RL by 10.4%, while the cycles with ascorbic acid promoted an increase of 14.2%, albeit not differing from Sempre Verde when compared to salt stress with no priming cycles.

Figure 2 - Mean values for first germination count (FGC), germination (G), shoot length (SL) and root length (RL) in seedlings of the cowpea varieties Sempre Verde and Pingo de Ouro subjected to elicitors of salt stress tolerance in three priming cycles (PC). Water (H_2O), gibberellic acid (GA_3), hydrogen peroxide (H_2O_2), salicylic acid (SA) and ascorbic acid (ASC). Mean values followed by the same lowercase letter (treatments) do not differ by Scott-Knott test at 5% probability, and followed by the same uppercase letter (varieties) do not differ by Student's t-test at 5% probability

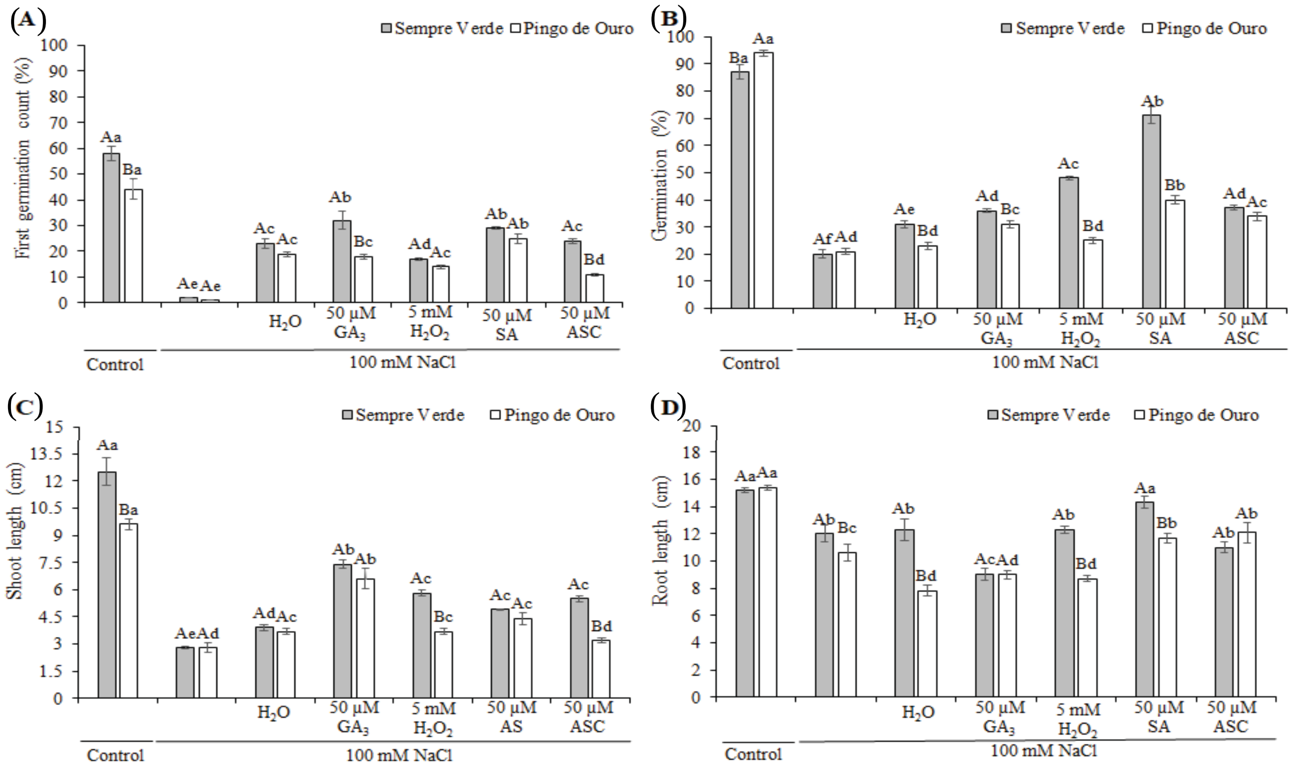
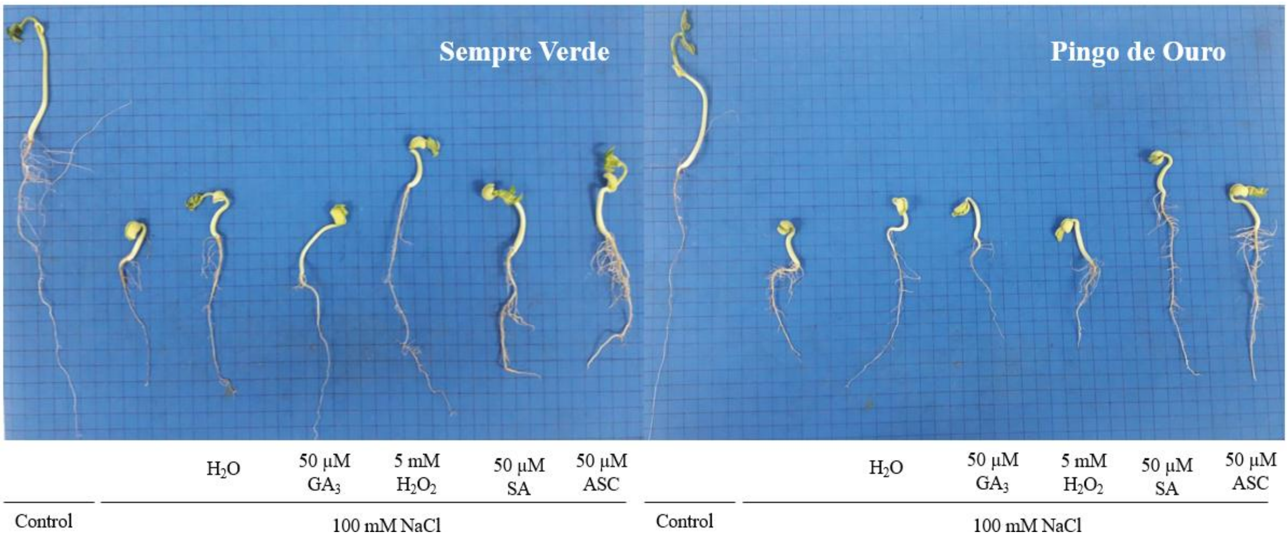


Figure 3 - Morphological aspects of seedlings of the cowpea varieties Sempre Verde and Pingo de Ouro subjected to elicitors of salt stress tolerance in three priming cycles. Water (H_2O), gibberellic acid (GA_3), hydrogen peroxide (H_2O_2), salicylic acid (SA) and ascorbic acid (ASC)



The suppressive effect of salt stress occurs in response to an excess of Na^+ and Cl^- ions, which causes toxicity to plant cells and is associated with a reduction in the levels of phytohormones produced by plants, and which are responsible for mediating defence responses (Verma; Ravindran; Kumar, 2016).

Salt stress causes the inactivation of genes related to the production of gibberellin, a plant hormone responsible for promoting germination and favouring seedling growth (Liu *et al*., 2018). This explains the reduction in germination and seedling length in the Sempre Verde and Pingo de Ouro varieties. Praxedes *et al.* (2020) found that a salinity of 5.5 dSm^{-1} did not reduce germination potential, but affected the parameters of seedling growth. The initial length of the cowpea is therefore affected under salt stress, resulting in a lack of stand uniformity, and compromising the development of the crop.

Applying an eliciting agent of salt stress tolerance in priming cycles reduced the physiological parameters of the seedlings of both cowpea varieties under a salt stress of 100 mM, compared to the control. However, when comparing the results obtained in the priming cycles with elicitors of salt stress tolerance with those of salt stress with no priming cycles, there was an increase in the response of the cowpea varieties. This suggests that the elicitors of salt stress tolerance helped in acclimatising the cowpea varieties, with the response varying according to the variety, the eliciting agent and the variable under analysis.

Priming cycles with water favour the germination and initial development of seedlings of agronomic and forestry species that occur in the Caatinga ecosystem under water stress (Sarmento *et al*., 2020). Indeed, the results obtained for the cowpea varieties under salt stress reaffirm this finding due to the increase in germination potential seen from the first germination count onwards, followed by an increase in the shoots, total dry weight and tolerance index of the roots. These findings show that tolerance was acquired in the varieties under salt stress following the priming cycles with water, especially the Sempre Verde variety.

This response was probably influenced by the increase in tissue hydration from seed imbibition, as well as benefitting from previous periods of drought, which favoured earlier plant stress and acclimatisation (Nascimento; Dantas; Meiado, 2021; Sarmento *et al*., 2020), and germination in the face of the new stress (salt). This behaviour is possible in drought-tolerant species such as the cowpea.

The priming cycles with gibberellic acid stood out in promoting germination and length, and in reducing the negative effects of salt stress on the cowpea seedlings. The increase in shoot length in the Sempre Verde and Pingo de Ouro varieties after priming cycles with gibberellic acid may have been a response to an improvement in osmoregulation capability. This is possible due to the accumulation of compatible solutes in cowpea varieties that maintain membrane integrity by preventing lipid peroxidation, reducing the effects of reactive oxygen species produced under stress (Miri *et al*., 2021; Pereira *et al*., 2023).

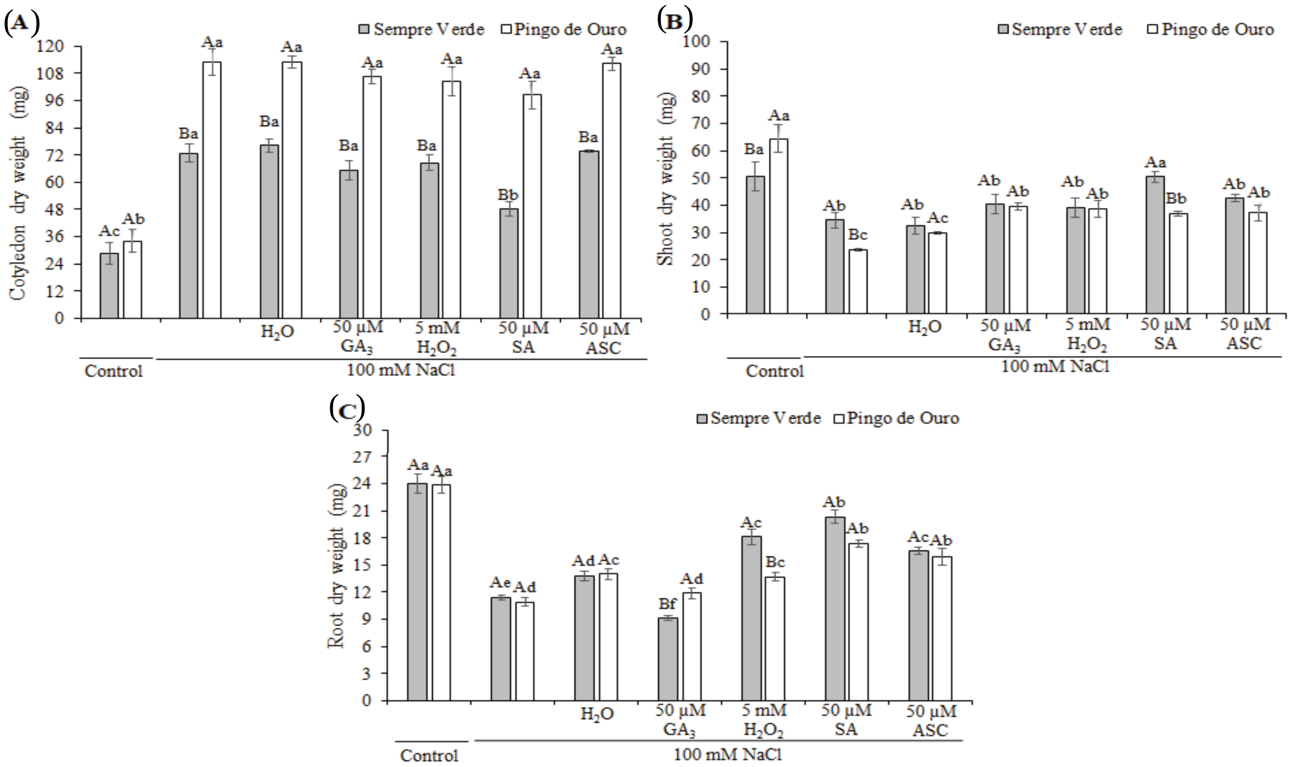
The priming cycles with salicylic acid favoured root length in both varieties under salt stress. This result may be due to the reduction in intracellular Na^+ accumulation promoted by this acid, which favours ionic balance (Na^+/K^+), and which is the reason for the recovery in length, especially of the roots (Liu *et al*., 2022). In studies carried out by Araújo *et al*. (2018) on cowpea seeds, and Pereira *et al*. (2023) on maize, each found that priming with salicylic acid favours tolerance to osmotic stress during germination and the early growth stages of the seedlings.

Each of the salt treatments reduced the consumption of cotyledon reserves in both varieties. Sempre Verde obtained an average of 71.28 mg seedling^{-1} cotyledon dry weight, except in the priming cycles with salicylic acid, which resulted in 48.2 mg seedling^{-1} cotyledon dry weight. For Pingo de Ouro, the average result was 107.85 mg seedling^{-1} cotyledon dry weight (Figure 4A).

Salt stress with no priming cycles promoted a reduction in shoot dry weight of 31.7% and 63.2% for the Sempre Verde and Pingo de Ouro varieties, respectively. For Sempre Verde, the priming cycles with salicylic acid promoted results similar to the control, with a gain of 45.7% compared to salt stress with no priming cycles (Figure 4B). For Pingo de Ouro on the other hand, only the priming cycles with water showed a similar response for shoot dry weight in relation to salt stress with no priming cycles. Also for this variety, shoot dry weight was on average 60.4% higher for the priming cycles with gibberellic acid, hydrogen peroxide, salicylic acid and ascorbic acid.

Root dry weight accumulation in seeds exposed to salt stress with no priming cycles was reduced by 52.5% and 54.2% compared to the control, in Sempre Verde and Pingo de Ouro, respectively (Figure 4C). The priming cycles with tolerance elicitors favoured root dry weight in Sempre Verde, particularly the cycles with salicylic acid, with an increase of 78.1% compared to salt stress with no priming cycles. For this variety, the smallest root dry weight accumulation (62.1%) occurred in the priming cycles with gibberellic acid. A similar result was obtained for Pingo de Ouro. The priming cycles with gibberellic acid did not promote any gains in root dry weight compared to salt stress with no priming cycles. However, the priming cycles with salicylic acid and those with ascorbic acid resulted in more root dry weight accumulation, of 59.6% and 45.9%, respectively, compared to salt stress with no priming cycles.

Figure 4 - Mean dry weight of the cotyledons (COTDW), shoots (SDW) and roots (RDW) in seedlings of the cowpea varieties Sempre Verde and Pingo de Ouro subjected to elicitors of salt stress tolerance in three priming cycles (PC). Water (H_2O), gibberellic acid (GA_3), hydrogen peroxide (H_2O_2), salicylic acid (SA) and ascorbic acid (ASC). Mean values followed by the same lowercase letter (treatments) do not differ by Scott-Knott test at 5% probability, and followed by the same uppercase letter (varieties) do not differ by Student's t-test at 5% probability



The interaction between the cowpea varieties and the pre-germination treatments was significant for the salt tolerance index of the shoots and roots, and when quantifying the total soluble sugars, amino acids ($p < 0.01$) and proline ($p < 0.05$) (Table 3).

The salt tolerance index of the shoots showed that the Sempre Verde variety is moderately tolerant (60% < STI < 80%) to a salinity of 100 mM NaCl, while Pingo de Ouro was sensitive (STI < 40%) (Figure 5A). After the priming cycles with salicylic acid and ascorbic acid, Sempre Verde became tolerant (STI > 80%). Pingo de Ouro obtained lower results for the salt tolerance index of the shoots compared to Sempre Verde, however, after the priming cycles with the tolerance elicitors, it became moderately sensitive to salinity (40% < STI < 60%). The salt tolerance index of the roots of both varieties decreased when the seeds were sown under salt stress with no priming cycles. Sempre Verde and Pingo de Ouro went from moderately sensitive to salinity (40% < STI < 60%) to moderately tolerant (60% < STI < 80%) following priming cycles with salicylic acid and ascorbic acid.

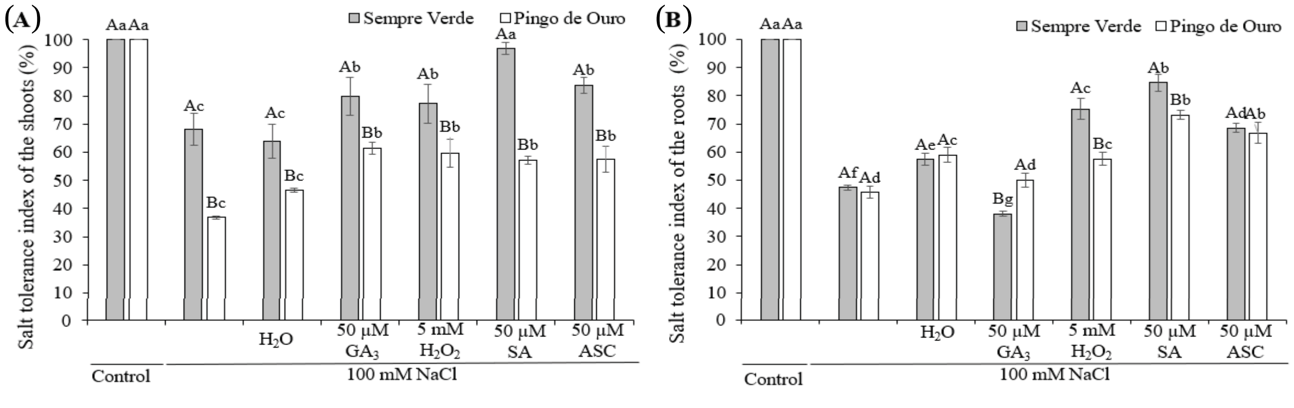
The highest levels of total soluble sugars (TSS) occurred in the control treatment for Sempre Verde, with a reduction of 12.4% following salt stress with no priming cycles. The priming cycles with gibberellic acid and those with ascorbic acid led to an increase in the accumulation of sugars in this variety, giving a similar result to the control (Figure 6A). When Sempre Verde was subjected to priming cycles with salicylic acid, the sugar accumulation was reduced by 23.4% compared to the control, while in the Pingo de Ouro variety, there was a reduction in sugar accumulation of 45.6% compared to the control. Priming cycles with hydrogen peroxide promoted a greater accumulation of

Table 3 - Analysis of variance for the salt tolerance index of the shoots (STIS) and roots (STIR), total soluble sugars (TSS), amino acids (AA) and proline (PRO) in seeds of the cowpea varieties Sempre Verde and Pingo de Ouro subjected to priming cycles with elicitors of salt stress tolerance

SV	DF	Mean square				
		STIS (cm)	STIR (cm)	TSS (mg TSS/ g MF)	AA (µmol AA/ g FM)	PRO (µmol PRO/g FM)
Treatments	6	1971.12**	2992.33**	127.77**	890.76**	6.58**
Cultivars	1	6510.5**	103.41*	32.89**	1175.47**	30.29**
Treat. x Cult.	6	318.68**	1095.85**	57.67**	27163**	0.76*
Error	42	65.03	20.06	1.94	13.96	0.27
CV (%)		11.14	6.79	7.14	5.35	11.57
Mean value		70.65	65.97	19.54	69.78	4.53

SV: source of variation; GL: degrees of freedom; **; *: significant effect at 0.01 and 0.05 significance

Figure 5 - Salt tolerance index of the shoots (STIS) and roots (STIR) in seedlings of the cowpea varieties Sempre Verde and Pingo de Ouro subjected to salt stress tolerance elicitors in three priming cycles (PC). Water (H_2O), gibberellic acid (GA_3), hydrogen peroxide (H_2O_2), salicylic acid (SA) and ascorbic acid (ASC). Mean values followed by the same lowercase letter (treatments) do not differ by Scott-Knott test at 5% probability, and followed by the same uppercase letter (varieties) do not differ by Student's t-test at 5% probability



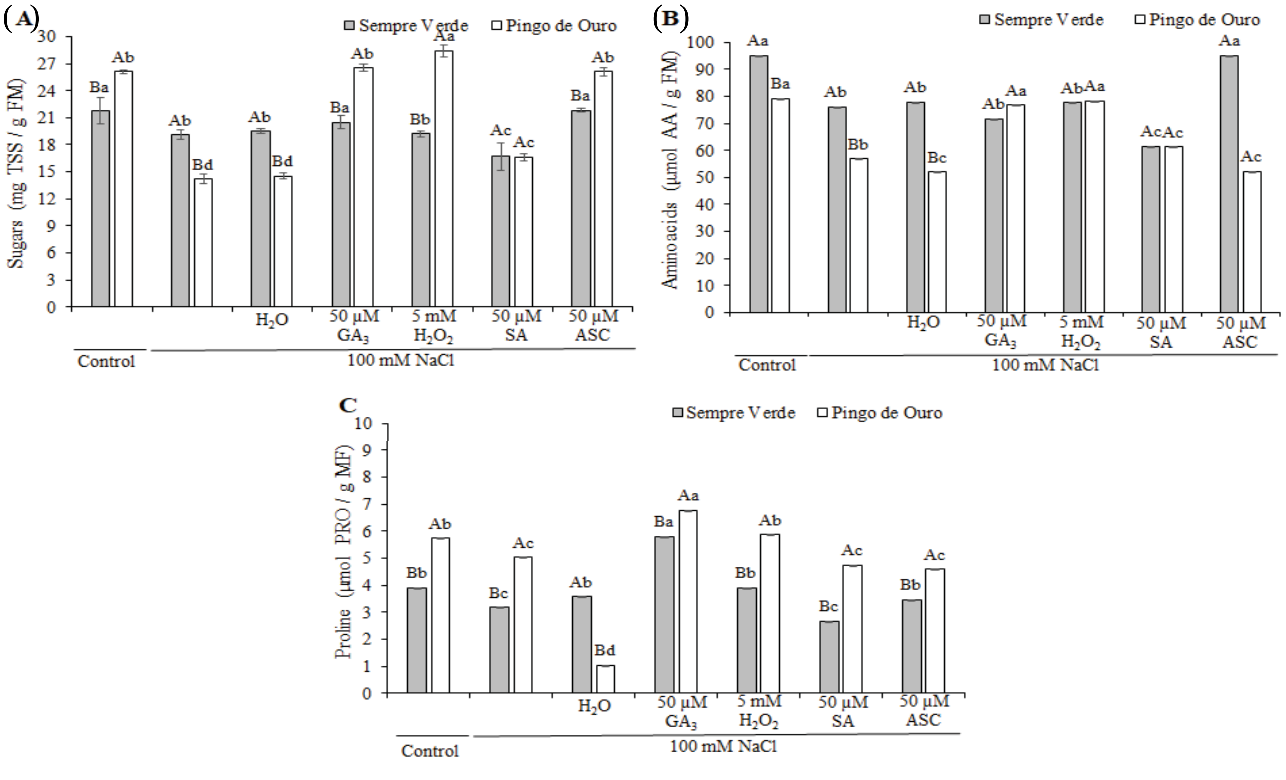
sugars than the control; on the other hand, the priming cycles with water and those with salicylic acid reduced sugar accumulation by 44.1% and 36.4%, respectively.

The amino-acid concentration was reduced when the cowpea varieties were subjected to salt stress with no priming cycles, by 20% and 28.2% for Sempre Verde and Pingo de Ouro, respectively (Figure 6B). In Sempre Verde, the priming cycles with ascorbic acid promoted a higher concentration of amino acids (25%) compared to salt stress with no priming cycles, showing no difference to the control. In Pingo de Ouro, the priming cycles with gibberellic acid and those with hydrogen peroxide favoured the concentration of amino acids, with similar results to the control. In Pingo de Ouro, the lowest concentrations of amino acids occurred in the priming cycles with water (34.2%) and ascorbic acid (33.9%), compared to the control.

The proline concentration decreased by 18.9% for Sempre Verde and 12.2% for Pingo de Ouro when subjected to salt stress with no priming cycles (Figure 6C). Priming cycles with tolerance elicitors favoured an increase in proline concentration, especially for Sempre Verde in cycles with gibberellic acid, with an increase of 83.3% compared to salt stress with no priming cycles. Priming cycles with salicylic acid reduced the proline concentration, as did salt stress with no cycles. Priming cycles with gibberellic acid promoted an increase of 34.5% in the proline concentration for Pingo de Ouro, followed by hydrogen peroxide, with a gain of 16.7%, compared to salt stress with no priming cycles. In the same variety, the other tolerance elicitors resulted in proline concentrations similar to those under salt stress with no priming cycles.

The results for homeostasis show that there is no difference between salt stress with no priming cycles and cycles with water when quantifying sugars and amino acids in the two varieties. Increasing proline production is a plant response to stress, verified in Sempre Verde, where the cycles with water afforded greater tolerance to salt stress. Pingo de Ouro may have used another strategy to acclimatise to the excess of salts, increasing the production of H_2O_2, which in excess can cause cell death. In order to keep their metabolism active, plants tend to produce antioxidant enzymes e.g. superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) (Ali *et al*., 2021). This response favours germination and the resumption of seedling growth, since it catalyses the breakdown of H_2O_2 into H_2O and O_2, corroborating the results of Araújo *et al*. (2017) in varieties of the cowpea.

Figure 6 - Mean-value test for total soluble sugars (TSS), amino acids (AA) and proline (PRO) in seedlings of the cowpea varieties Sempre Verde and Pingo de Ouro subjected to salt stress tolerance elicitors in three priming cycles (PC). Water (H_2O), gibberellic acid (GA_3), hydrogen peroxide (H_2O_2), salicylic acid (SA) and ascorbic acid (ASC). Mean values followed by the same lowercase letter (treatments) do not differ by Scott-Knott test at 5% probability, and followed by the same uppercase letter (varieties) do not differ by Student's t-test at 5% probability



Among the osmoprotectants quantified for cowpea varieties, priming cycles with gibberellic acid, compared to salt stress with no priming cycles, promoted higher levels of sugars and proline in the Sempre Verde and Pingo de Ouro varieties, including other amino acids in Pingo de Ouro. Osmoregulation promoted by gibberellic acid causes a reduction in water potential in cowpea seedlings, ensuring the resumption of water absorption and preventing dehydration of the tissue (Araújo *et al*., 2017). Pingo de Ouro proved to be less tolerant to a salt stress of 100 mM NaCl. However, following the priming cycles with gibberellic acid, this variety produced the most osmoprotectants, indicating an attempt to acclimatise to the salt stress.

The priming cycles with hydrogen peroxide under salt stress resulted in better germination than salt stress with no priming cycles for both cowpea varieties. Sempre Verde stood out with germination above 70%, in addition to promoting greater shoot and root length than Pingo de Ouro. Hydrogen peroxide activates physiological mechanisms that increase the salt stress tolerance of the plants by reducing Na^+ and Cl^- ions in the leaves. This helps maintain nutrient translocation and absorption so that germination occurs in response to the reduction in oxidative damage due to increased enzyme activity (Silva *et al*., 2022).

The results of the homeostasis due to the accumulation of osmoprotectants show that the priming cycles with salicylic acid were not responsible for the greater tolerance of Sempre Verde. The strategy adopted by this variety was to remobilise the reserves present in the cotyledons, which favoured seedling length and a greater accumulation of shoot and root dry weight when produced under a salt stress of 100 mM NaCl, affording greater tolerance. In Pingo de Ouro, the priming cycles with stress tolerance elicitors did not result in the remobilisation of cotyledonary reserves, which may have reflected in the lower tolerance of this variety to salt stress of 100 mM NaCl compared to Sempre Verde.

The priming cycles with ascorbic acid allowed the cowpea varieties to develop more vigorously under salt stress in response to the regulation of cellular homeostasis (Chen; Cao; Niu, 2021) by the accumulation of sugars and, especially, amino acids. This can be seen from the greater values for germination, root length and shoot dry weight in both varieties, especially Sempre Verde, due to the greater length of the shoots. The production of osmoprotectants such as sugars and amino acids, together with the reduction in damage to the cytoplasmic membranes caused by oxidative stress following the priming cycles with ascorbic acid, contributed to the positive response of the cowpea varieties to salt stress (Nunes *et al*., 2019).

The tolerance acquired by the cowpea varieties, for the most part, was possible due to osmotic homeostasis from the accumulation of solutes compatible with the plant metabolism; however, plants can adopt other strategies, as shown by the results for the cowpea varieties. The different responses show the importance of studies on the action of agents that elicit stress tolerance on plant morphophysiology and metabolism. Knowledge of tolerance acquisition in this species is important as it is sown directly in the field and does not always find environmental conditions that favour germination and initial seedling development, particularly due to water restrictions caused by excess salts in the soil.

CONCLUSIONS

Salt stress (100 mM NaCl) reduces germination, length, and biomass accumulation in the Sempre Verde and Pingo de Ouro varieties of the cowpea. Priming cycles with gibberellic and salicylic acids promoted greater germination potential, length and biomass under a salt stress of 100 mM NaCl, affording greater tolerance thanks to osmotic regulation, especially in the Sempre Verde variety.

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