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Physiological potential of genipap seed and in vitro growth of accessions under saline stress

Abstract – The objective of this work was to evaluate the effect of saline stress on the physiological potential of seed and on in vitro growth of three genipap accessions. Seed of accessions NB (Núcleo Bandeirante, DF), SA (Sabinópolis, MG) and CRA (Cruz das Almas, BA) were subjected to NaCl concentrations (at 0, 25, 50, 75, and 100 mmol L^{-1}) for 40 days. The germination was evaluated until seedling formation. Every 72 hours, embryonic axes of seed subjected to saline concentrations were extracted, to determine the enzyme activity of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). To evaluate the initial growth, explants of the accessions were inoculated in MS medium added with the same NaCl concentrations and, at 30, 60, and 90 days, proline was quantified. The concentration at 50 mmol L^{-1} NaCl increased the SOD and APX activity up to 288 hours of exposure to saline stress. The SA accession showed the greatest tolerance to saline stress, with proline increase at 30, 60 and 90 days. Saline stress affects the genipap germination and its initial in vitro growth with varying degrees of tolerance among accessions, for which SA showed the highest degree.

Index terms: *Genipa americana*, antioxidant enzymes, Brazilian fruit, proline.

Potencial fisiológico da semente de jenipapo e crescimento in vitro sob estresse salino

Resumo – O objetivo deste trabalho foi avaliar o efeito do estresse salino sobre o potencial fisiológico de sementes e sobre o crescimento in vitro de três acessos de jenipapeiro. Sementes dos acessos NB (Núcleo Bandeirante, DF), SA (Sabinópolis, MG) e CRA (Cruz das Almas, BA) foram submetidas às concentrações de NaCl a 0, 25, 50, 75 e 100 mmol L^{-1} por 40 dias. A germinação foi avaliada até a formação de plântula. A cada 72 horas, eixos embrionários das sementes submetidas ao NaCl foram extraídos para a determinação da atividade das enzimas superóxido dismutase (SOD), catalase (CAT) e ascorbato peroxidase (APX). Para avaliação do crescimento inicial, explantes dos acessos foram inoculados em meio MS, acrescido das mesmas concentrações de NaCl e, aos 30, 60 e 90 dias, a produção de prolina foi quantificada. Verificou-se que na concentração a 50 mmol L^{-1} de NaCl, houve aumento da atividade das enzimas SOD e APX até 288 horas de exposição ao estresse salino. O acesso SA apresentou a maior tolerância ao estresse salino, com incremento de prolina aos 30, 60 e 90 dias. A germinação e o crescimento inicial in vitro do jenipapeiro são afetados pelo estresse salino, com grau de tolerância variado entre acessos, em que SA obteve o maior grau.

Termos para indexação: *Genipa americana*, enzimas antioxidantes, frutas brasileiras, prolina.

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Introduction

Genipap (*Genipa americana* L., Rubiaceae) is a tropical fruit tree, still in the domestication phase, that has aroused the interest of the scientific community due to its ecological, social, and economic importance (Silva et al., 2020; Silva et al., 2024). It was included in a list of 39 priority species in the 'Plants for the Future' program in FAO International Treaty on Plant Genetic Resources for Food and Agriculture (Coradin et al., 2018). Genipap is indicated as one of the 15 priorities for silvicultural research and plantings in the Atlantic Forest (Rolim et al., 2019).

It is a late secondary species of tolerant to moderate shading when young, and with intense regeneration in areas with anthropogenic activities. Its seed are considered intermediate, and support reductions of the water content from 7% to 10%, with loss of viability at values below these (Magistralli et al., 2013). Over time, it began to be indicated in recovery programs for degraded areas. It is considered to have a broad potential for use in the food, pharmaceutical, and cosmetic industries. Neves et al. (2022) reports the obtaining of natural blue dyes as one of the biggest challenges of the food coloring industry; in this case, genipap is a source of genipin, an iridoid that corresponds from 1% to 3% of the fruit composition. Advancing research on genipap is of crucial importance for preserving its genetic resources, to expand its use, and subsidize a future production system. Genipap iridoid compounds – genipin and geniposide – have an important role in toxicity, with high-repellency and low-persistence to *Aceria guerreronis* (Jesus et al., 2019; Jesus et al., 2020). The species is also used in urban afforestation, recovery of degraded areas, composition in permanent preservation areas, and in agroforestry systems (Sá et al., 2015; Sivano-Sànchez et al., 2023); it is also widely used by folk medicine (Santos et al., 2023).

Salinity is one of the main abiotic stresses, responsible for triggering several structural, physiological, and biochemical disturbances in tissues (Daliakopoulos et al., 2016), which can affect the processes of germination, vegetative growth, and reproductive development. In addition, changes in the cellular balance, caused by high salt concentration, can lead to an increase of the production of reactive oxygen species (ROS) to levels that are toxic to cells, among which are the superoxide radical ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). Consequently, they can cause oxidation of membrane lipids, proteins, and nucleic acids, leading to cell death (Zabeu et al., 2022).

In abiotic stress conditions, proline can trigger multiple responses that are part of the acclimation process because its accumulation favors osmotic adjustment, contributes to the stabilization of membranes and proteins, acts in cell signaling, promotes the elimination of free radicals, participates in the balance of oxireduction and in the induction of gene expression (Lehmann et al., 2010). In addition to proline, antioxidant enzymes such as superoxide dismutase, catalase, and peroxidase are part of the plant defense mechanism that assist in the elimination and neutralization of reactive oxygen species produced under salt stress (Naing & Kim, 2021).

Araújo et al. (2023) observed for hydric stress that the increase of mannitol concentrations resulted in the inhibition of adventitious shoot growth. The behavior of genipap accessions subjected to abiotic conditions is not fully known. The development of this work is important for conservation strategies of the genetic resources and breeding program of the species.

The objective of this work was to evaluate the effect of saline stress on the physiological potential of seed and on in vitro growth of three genipap accessions.

Materials and Methods

Genipap seed used in the experiment were extracted from some accessions of the Active Genipap Genebank of Embrapa Coastal Tablelands, located at the experimental station Jorge Sobral, in the municipality of Nossa Senhora das Dores (10°29'30"S, 37°11'36"W), in the state of Sergipe (SE), Brazil.

The experiments were carried out at the Laboratory of Plant Tissue Culture of Embrapa Coastal Tablelands, located in the municipality of Aracaju, SE. The genipap accessions used were NB (from Núcleo Bandeirante, Federal District (DF)); SA (from the municipality of Sabinópolis, state of Minas Gerais (MG)); and CRA (from the municipality of Cruz das Almas, state of Bahia (BA)).

The effect of saline stress on the physiological potential of seed and in vitro growth of genipap accessions were determined by evaluating the activity of antioxidant enzymes in the embryonic axes of seed and the initial growth of explants under NaCl concentrations, and quantifying proline production in accessions subjected to different times of NaCl concentration.

All trials were conducted in a completely randomized design. The trials with antioxidant enzymes were designed in a split-plot arrangement, for which the factor NaCl concentration (0, 25, 50, 75, and 100 mmol L^{-1}) constituted the plot, and the exposure time to salt (30, 60, and 90 days) the subplot, with two replicates. In vitro growth trials were performed in a 3 × 5 factorial arrangement (accessions NB, SA, and CRA; and NaCl concentrations 0, 25, 50, 75, and 100 mmol L^{-1}), with seven replicates consisting of three plants. For proline quantification, a split-plot design was used, with the factor accessions (NB, SA, CRA) and NaCl concentrations (0, 25, 50, 75, and 100 mmol L^{-1}) in the plot, and the time in the subplot.

To simulate salt stress, genipap seed were sown on two sheets of germitest paper, in gerbox, moistened with treatments 0, 25, 50, 75, and 100 mmol L^{-1} of NaCl, in a volume 2.5 times the weight of the paper. The boxes were placed in a biochemical oxygen demand (BOD) germination chamber equipped with fluorescent lamps, under a constant temperature of 28±2°C, and under dark conditions for 40 days. Seed germination under these treatments were performed daily by counting the beginning of root protrusion and evaluations until the formation of seedlings (Brasil, 2009). Seed viability was evaluated by means of the germination percentage (G), taking into account the formation of normal seedlings as follows: G (%) = (N/A) × 100, in which: G is the germination percentage; N, is the number of germinated seed; and A is the total number of seed put to germinate. The counts started on the 8^{th} day after sowing in germitest, and the germination percentage was calculated on the 40^{th} day after sowing, when the emergence of the plants stabilized. The results were transformed into angular value, by the equation (arc sen √ x/100), in order to meet the normality criteria. Seed vigor was assessed by the germination speed index (GSI) (Maguire, 1962), as follows: GSI = G1/N1 + G2/N2 + ... + Gn/Nn, in which: G is the number of germinated seedlings computed in the first, second, and last count; and N is the mean germination time, which is the number of days from sowing to first, second, and last (n) count. The mean germination time (MGT) was calculated by the following expression: MGT = ∑(ni.ti)/∑ni, in which: (ni) is the number of seed germinated in a time interval; (ti) is the germination time interval. The mean germination speed (MGS) is calculated by 1/MGT.

The previously described embryonic axes from seed subjected to the NaCl concentrations were excised every 72 hours, and frozen at -80°C until the moment of evaluation. To determine the activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), crude enzyme was extracted by macerating approximately 0.1 g of the embryos in 1.5 mL of potassium phosphate extraction buffer solution (0.1 mol L^{-1}, pH 7.0), containing 0.1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA) and 1% (w/v) polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 14.000 × *g* for 20 min at 4°C.

Superoxide dismutase activity was determined by adding 100 µL of the crude enzyme extract to 3 mL of the reaction medium, which consisted of 50 mmol L^{-1} potassium phosphate buffer (pH 7.8) and 0.1 mmol L^{-1} of EDTA. Superoxide generator system – containing 14.3 mmol L^{-1} of methionine, 82.5 mmol L^{-1} of p-nitro tetrazolium blue (NBT), and 2.2 µmol L^{-1} of riboflavin – was added to the reaction medium. The reaction took place at 25±2°C in a BOD-type germination chamber equipped with four 20 W fluorescent lamps. The tubes were kept 30 cm below the light source for 15 min and, after that time, the lamps were turned off, and the blue formazan produced by photoreduction of NBT was measured by absorbance at 560 nm. The absorbance value of all reagents – together with the crude enzyme extract kept in the dark for the same period – served as the blank and was subtracted from the result of each sample that received light. Enzyme activity was expressed as a SOD unit, with 1 SOD defined as the amount of enzyme required to inhibit about 50% NBT under the assay conditions (Beauchamp & Fridovich, 1971).

Catalase activity was determined by adding 100 µL of the crude enzyme extract to 2 mL of the reaction buffer, which was composed of 50 mmol L^{-1} of potassium phosphate buffer pH 7 and 10 mmol L^{-1} of H_2O_2. Enzyme activity was determined by monitoring the decrease in absorbance at 240 nm (ε = 36 per mmol L^{-1} cm^{-1}), for 2 min, at 15 s intervals. The reduction rate of the assay (ΔA) was used to determine the enzyme activity (expressed as µmol min^{-1} g^{-1}).

The peroxidase activity was determined according to Nakano & Asada (1981). The reaction solution

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with a total of 2 mL was composed of 50 mmol L^{-1} of sodium phosphate buffer (pH 7.0), 0.1 mmol L^{-1} of EDTA, 0.25 mmol L^{-1} of sodium ascorbate, 1 mmol L^{-1} of H_2O_2, and 100 µL of the crude enzyme extract. The H_2O_2-dependent ascorbate oxidation was measured by decreasing absorbance at 290 nm ($\varepsilon = 2.81$ per mmol L^{-1} cm^{-1}), for 5 min, every 30 s. The activity of ascorbate peroxidase was expressed as (µmol min^{-1} g^{-1}).

One-centimeter microcuttings containing a single bud, which were taken from pre-established plants and multiplied in vitro, were transferred to glass vials containing 30 mL of MS medium (M5519, Sigma-Aldrich, St. Louis, Missouri, EUA). The medium was supplemented with 30 g L^{-1} of sucrose, and different concentrations of salts (0, 25, 50, 75 and 100 mmol L^{-1} NaCl), then solidified with 3.5 g L^{-1} of Phytagel (Sigma, P8169), pH was adjusted to 5.8 and, then, the solution was subjected to sterilization in an autoclave at 120±1°C for 20 min. After bud transferring, the containers were capped and transferred to a growth room at 25±2°C, with average relative humidity about 70%, and a 12-hour photoperiod with 60 µmol m^{-2} s^{-1} light intensity. After 90 days, the following variables were evaluated: number of roots, number of leaves, number of shoots, seedling length (measured from the root to the hypocotyl, using a graduated ruler), number of buds, stem diameter (measured with a digital caliper), and mass of shoot dry matter (determined using a precision balance).

Proline quantification was determined by the protocol of Bates et al. (1973), which was modified by replacing filtration with centrifugation of the extracts. Proline concentration (expressed in µmol g^{-1}) tests were performed in duplicate.

Statistical analyses were performed as follows. For germination under NaCl concentrations, the analysis of variance using polynomial regression analysis ($p<0.05$) was used, with polynomial regressions fitted for the salt treatments. In the antioxidant enzyme evaluations for NaCl levels, the regression test was used ($p<0.05$) and, for time in NaCl concentration, the Tukey's test was employed ($p<0.05$). For in vitro growth evaluations, NaCl levels were analyzed by regression test, and the accessions were compared by Tukey's test ($p<0.05$). In proline quantification, for salt treatments, the regression test with polynomial regressions was used, and for time in NaCl concentration and accessions, the means were compared by Tukey's test ($p<0.05$).

Results and Discussion

The percentage of germination decrease in genipap seed was directly proportional to the increase of salinity, with the 100 mmol L^{-1} concentration affecting seed germination the most (Figure 1). In many species, salt stress inhibits germination and initial growth, due not only to reduced water potential, but also to changes in seed metabolism, which can lead to inhibition of reserve mobilization and changes in the membranes of the embryonic axes (Marques et al., 2011). Thus, the presence of NaCl entails reductions of water potential, hindering the soaking process by the seed,

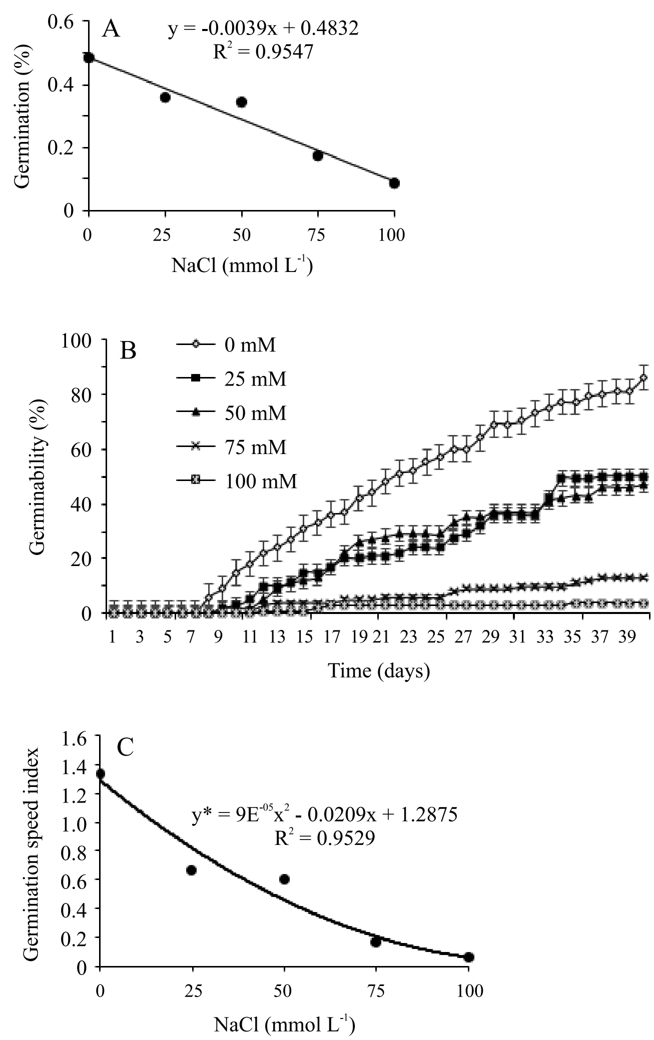

Figure 1. Genipap (*Genipa americana* L., Rubiaceae) seed subjected to NaCl concentrations in vitro: A, germination percentage; B, average germinability; and C, germination speed index.

resulting in a decrease of the germination process. In addition, there is also the ingress of ions present in the salt, which cause toxicity to the embryo and/or to endosperm membrane cells (Voigt et al., 2009).

The distribution of germination over time reinforces the significant effect of salinity on the germinability of genipap seed (Figure 1). The time for seed to start germination increased with increasing salt concentrations. For the control treatment, it was found that germination onset occurred on the 7th day, and germination stabilization occurred between the 37th and 39th day after sowing. For the concentrations of 25 and 50 mmol L^{-1}, the time for onset varied between 9 and 11 days, and stabilization was achieved between 35 and 37 days after sowing. For the 100 mmol L^{-1} concentration, there was a more critical result with later germination, starting at 15 days after sowing, which made it even more evident that salinity interfered in the germination process of genipap seed.

Seed vigor was affected also by the decrease of osmotic potential; and for the germination percentage, the higher concentration of NaCl resulted in a lower index of speed germination (Figure 1), and 0.0741 was its minimum value. One of the first characteristics to be affected in the process of seed deterioration is the speed of germination; thus, the reduction of the germination power, when compared to the control treatment, indicates that salinity interfered in the germination and vigor of seed, justifying the species degree of tolerance to salinity. The reductions of germination percentage, germination speed index, and the average increase of germination time may also be related to the fact that genipap seed are of intermediate successional stage.

There was a significant interaction between NaCl concentrations and the immersion times (Table 1).

Table 1. Activity of antioxidant enzymes in seed of genipap (*Genipa americana* L., Rubiaceae) subjected to NaCl concentrations, at different time periods, in vitro[(1)].

Time (hours)	NaCl concentrations (mmol L^{-1})					Regression	R^2
	0	25	50	75	100		
			Superoxide dismutase – SOD (SOB unit)				
0	1.632a	1.522b	1.478a	1.507a	1.669a	$y^{**}= 0.00007x^2 - 0.0068x + 1.6384$	0.9828
72	0.990b	1.155c	1.204c	1.165b	1.368b	$y^{**}= 0.0031x + 1.0237$	0.8052
144	1.709a	1.036d	1.551a	1.110b	1.507a	$y^{**}= 0.0001x^2 - 0.0148x + 1.6178$	0.3216
216	1.816a	1.210c	1.408ab	1.491b	1.524a	$y^{*}= 0.0001x^2 - 0.0145x + 1.7165$	0.5486
288	1.635a	1.560b	1.505a	1.627a	1.699a	$y^{**}= 0.00005x^2 - 0.0046x + 1.6336$	0.8836
360	1.773a	1.856a	1.621a	1.508a	1.632a	$y^{**}= -0.0025x + 1.8043$	0.5298
CV (%)			4.03				
			Ascorbate peroxidase – APX (µmol min^{-1} g^{-1})				
0	0.792a	0.725ab	0.740a	0.681b	0.666b	ns	ns
72	0.501ab	0.688ab	0.691a	0.718b	0.747b	ns	ns
144	0.510ab	0.458b	0.644ab	0.376b	0.469b	ns	ns
216	0.441ab	0.491b	0.295bc	0.437b	0.754b	$y^{*} = 0.0001Ex^2 - 0.0077x + 0.4942$	0.7714
288	0.328b	0.916a	0.673ab	1.238ab	1.140a	$y^{**} = 0.0078x + 0.4703$	0.6977
360	0.485ab	0.385b	0.127c	0.464b	0.367b	ns	ns
CV (%)			20.90				
			Catalase – CAT (µmol min^{-1} g^{-1})				
0	0.454b	0.457c	0.460b	0.422a	0.458a	ns	ns
72	0.321b	0.256b	0.051a	0.102b	0.128b	ns	ns
144	0.481b	0.152ab	0.032a	0.187bc	0.117b	ns	ns
216	0.011a	0.019a	0.026a	0.028c	0.034bc	ns	ns
288	0.013a	0.021a	0.015a	0.025c	0.044bc	ns	ns
360	0.005a	0.006a	0.010a	0.015c	0.013c	ns	ns
CV (%)			20.69				

[(1)]Means followed by equal letters in the columns do not differ, by Tukey's test, at 5% probability. * and **Significant at 5% and 1% probability, respectively. [ns]Nonsignificant.

The enzymatic activity decreased after 72 hours and returned to the initial value at the final time. Only at 25 mmol L^{-1} concentration, there were differences at times 0 and 360 hours.

After the exposure to increasing salt stress, the production of ROS was intensified due to a low-CO_2 concentration, as a function of stomatal closure, and the limitation of CO_2 fixation in the Calvin cycle. This exposure causes an oxidation decrease of NADPH (nicotinamide adenine dinucleotide phosphate), and takes the electron from ferredoxin to O_2, forming superoxide radicals ($O_2^{\bullet-}$), which must be eliminated to avoid oxidative stress. Thus, the synchronized action of the enzymes responsible for the removal of ROS confers a greater protection and tolerance of the species to stress conditions. The first line of defense is SOD, by carrying out the dismutation of the radical to hydrogen peroxide (H_2O_2), justifying its higher activity in relation to the other evaluated enzymes.

For APX, there was no significant difference between treatments up to 144 hours after the week, when there was an activity increase from 216 hours onwards, at 75 mmol L^{-1} and 100 mmol L^{-1} NaCl concentrations (Table 1). This fact can be explained by the increased exclusion of H_2O_2 produced by SOD. The corresponding means for CAT activity were similar among the treatments and times tested. The CAT enzyme is important in the initial stages of germination, for removing the H_2O_2 produced during the β-oxidation of fatty acids. In general, its activity was lower than that of APX, justified by its smaller layer per H_2O_2. The APX and CAT pathways are simultaneous, which can result in the low activity of both. The cellular detoxification, by removing H_2O_2 and forming water and oxygen, is performed by these enzymes that belong to different groups, due to their hydrogen peroxide moisture layer, with APX (μmol L^{-1}) and CAT (mmol L^{-1}) (Veloso et al., 2022).

Water uptake by seeds is essential for antioxidant enzyme activities and, at the same time, activates in stressed seeds the metabolic pathways that contribute to the generation and accumulation of ROS (Wojtyla et al., 2016). Lipid peroxidation due to high-salt concentrations results in the formation of malondialdehyde (MDA), which characterizes damage to cell membranes and, as a consequence, the inactivation of antioxidant enzymes by the excessive accumulation of free radicals, causing a potential oxidative damage and cell death by exposure to NaCl. Such factors may explain the low activity of enzymes in this study and the reduced germination potential of the seed.

Salt concentrations and accessions influenced the number of root, leaf, shoot, and bud (Table 2). Only the salt treatments were significant for stem diameter. The interaction of the factors (salt concentration x genotype) significantly influenced root size, plant length, and the mass of dry matter, affecting the in vitro growth of explants of genipap tree. The number of roots decreased with the salt increment to the medium, going from 0.73 roots on average in the control treatment to 0.29 roots at the 100 mmol L^{-1} concentration. Between the studied accessions, SA showed the highest average root production (0.857 per plant), while CER and CRA obtained 0.297 and 0.369 per plant, respectively, leading to a reduction of 65% and 56%, approximately.

Salt stress causes nutrient imbalance, which together with osmotic stress and ion toxicity result in decreased growth. Salinity induces osmotic stress, which reduces water uptake. To avoid dehydration, plants need to activate tolerance mechanisms and can produce more roots and longer roots, to maximize water uptake, which is what happened in the present study.

Plant length decreased with increasing NaCl concentration (Table 2). The SA accession obtained an average seedling length of 9.96 cm in the control treatment, reaching an average of 3.25 cm in the presence of 100 mmol L^{-1} NaCl concentration, while the CRA accession went from 8.03 cm in the 0 mmol L^{-1} treatment to 1.85 cm in the 100 mmol L^{-1} of NaCl concentration. The SA stood out in all treatments in comparison with the other genotypes; and the NB accession obtained the lowest averages. Decreased plant length is a consequence of severe osmotic stress, which is equivalent to water stress and a nutritional imbalance in plants, caused by a loss of ability to transport or compartmentalize Na^+ at levels that are not toxic to the cell (Radanielson et al., 2018).

The number of leaves also decreased with increasing salt concentrations, and the SA obtained the highest average, reaching 5.91, showing that this accession had the greatest tolerance to salinity when compared to the others (Table 2). The reduction in the number of leaves is a result of the decrease of water uptake, a direct consequence of the osmotic effect caused by salinity. Under stress conditions, these morphological

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changes may occur for their own survival. The SA accession, with 0.504 average, produced more shoots than the others did. The number of shoots is one of the most widely used variables to verify adaptation to the in vitro environment. Overall, the shoot production was the lowest one at 63.57 mmol L^{-1} concentration, with an average of 0.13 shoots. For the number of buds, the SA accession showed the greatest production, while the lowest average (3.67) was obtained at 208.33 mmol L^{-1} saline concentration. This suggests that there are diverse responses to salt stress among the accessions, with a consequent decrease in the production of young tissues, such as sprouts and buds, because the increased osmotic potential in the saline medium affected the uptake of water and nutrients by impairing the growth points and cell expansion.

Table 2. Number of roots, seedling length, number of leaves, number of shoots, number of buds, stem diameter, and mass of dry matter of three accessions of genipap (*Genipa americana* L., Rubiaceae) subjected to NaCl concentrations, in vitro[(1)].

Accession[(2)]	NaCl concentration (mmol L^{-1})					Means
	0	25	50	75	100	
			Number or roots			
NB	0.333	0.473	0.500	0.047	0.150	0.297b
CRA	0.619	0.550	0.350	0.286	0.048	0.369b
SA	1.333	0.714	0.761	0.667	0.809	0.857a
CV (%)			34.09			
			Seedling length (cm)			
NB	4.004c	2.895b	4.030a	2.762b	2.171a	3.252
CRA	7.8762b	6.943a	4.740a	2.967b	2.540a	4.932
SA	10.990a	6.995a	6.281a	5.495a	3.328a	6.607
CV (%)			32.15			
			Number of leaves			
NB	4.666	3.263	3.900	3.619	3.500	3.802b
CRA	5.762	3.950	3.950	3.857	3.047	4.116b
SA	7.952	5.952	5.571	5.095	5.000	5.914a
CV (%)			24.07			
			Number of shoots			
NB	0.381	0.052	0.150	0.190	0.100	0.178b
CRA	0.000	0.000	0.050	0.047	0.047	0.029b
SA	1.000	0.333	0.381	0.333	0.476	0.504a
CV (%)			29.58			
			Number of buds			
NB	5.000	5.000	5.200	3.809	4.250	4.643b
CRA	6.142	5.650	4.900	3.857	5.523	5.213b
SA	8.619	8.190	7.095	6.714	5.190	7.161a
CV (%)			21.71			
			Stem diameter (mm)			
NB	1.7614	1.7431	1.5980	1.6819	1.7140	1.6997b
CRA	1.8376	1.7115	1.834	1.7514	1.9600	1.8190b
SA	2.5419	2.0609	2.1647	1.9695	1.8890	2.1251a
CV (%)			39.86			
			Mass of dry matter (g)			
NB	0.0221b	0.0235a	0.0281a	0.0191b	0.0215a	0.0229
CRA	0.0292b	0.0206a	0.0228a	0.0205ab	0.0274a	0.0241
SA	0.0447a	0.0280a	0.0297a	0.0281a	0.0247a	0.0310
CV (%)			42.51			

[(1)]Means followed by equal letters in the columns do not differ, by Tukey's test, at 5% probability. [(1)]Brazilian genipap accessions: NB, from Núcleo Bandeirante (Distrito Federal-DF); CRA, from Cruz das Almas (Bahia state); SA, from Sabinópolis (Minas Gerais state).

The highest average for stem diameter (2.13 mm) was for the SA (Table 2). The reduction of growth variables was due to a decrease of cell division and elongation, caused by the accumulation of salts in the cell wall, decreasing turgor and, consequently, the growth of the individuals.

The highest mass of dry matter accumulation in the genotypes was for SA, in the 0 mmol L^{-1} and 75 mmol L^{-1} treatments (Table 2). The lowest mean dry matter mass was 0.029 g in the 66.66 mmol L^{-1} treatment for the SA, and mean 0.021 g for the 50 mmol L^{-1} salt concentration for CRA. With the overall decrease of growth variables, the dry matter decrease of seedling mass was expected, starting with the increase of NaCl in the medium. In strawberry (*Fragaria* spp., Rosaceae), increasing NaCl caused the mass decrease of aerial and root dry matter, the decreasing the survival among cultivars, and increasing Na^{+} concentration in the petiole and leaves of the plants studied (Ferreira et al., 2019). In pistachio cultivars, the salinity affected plant growth and development, causing necrosis and reduction of the leaf area, loss of chlorophyll, and reduction of aerial part length (Rahneshan et al., 2018). In the present work, a decrease of dry matter accumulation was also verified in genipap tree under saline conditions.

The three studied factors (time × NaCl concentrations × accessions) influenced the proline production in genipap tree explants subjected to salinity (Table 3). The NB accession showed an increase of proline production at 25 mmol L^{-1} (4.395 µm g^{-1}) and 50 mmol L^{-1} (4.370 µm g^{-1}) concentrations until 90 days, and, in more severe treatments (75 and 100 mmol L^{-1}), there was an increased production of amino acid (4.475 and 4.035 µm g^{-1}) until 60 days. However, NB did not show great tolerance to the presence of NaCl, a fact that is related to the decrease of plant growth.

The proline production in the CRA accession increased at the saline concentrations of 50 mmol L^{-1} (6.150 µm g^{-1}), 75 mmol L^{-1} (6.105 µm g^{-1}), and 100 mmol L^{-1} (3.040 µm g^{-1}) until 60 days. However, at 90 days, there was a production decrease of proline at all concentrations of NaCl, associated with a reduction of biometric variables (Table 3). In the SA accession, the most severe treatment (100 mmol L^{-1}) caused lower averages at 60 days (3.450 µm g^{-1}) and 90 days (4.260 µm g^{-1}). However, for growth data, this accession stood out. The SA accession obtained the highest proline production at 30 days, at 100 mmol L^{-1} saline concentration (5.266 µmol g^{-1}), while the NB accession expressed its lowest production at 70 mmol L^{-1} saline concentration, resulting in 2.39 µmol g^{-1} of amino acid. At the 60^{th} day, the maximum point of proline accumulation occurred in the CRA accession, at 34.35 mmol L^{-1} salt treatment with an average of 6.052 µmol g^{-1}, and for NB accession, the minimum production occurred at 44.14 mmol L^{-1} salt concentration, with 2.805 µmol g^{-1} average. At 90^{th} days, SA obtained the lowest average (2.400 µmol g^{-1}) at 51.3 mmol L^{-1} concentration, while NB achieved the highest average (4.673 µmol g^{-1}), at 48.3 mmol L^{-1} saline treatment. For the CRA accession, there was a decrease in the production of the amino acid because of NaCl increasing from 3.846 µmol g^{-1} at 0 mmol L^{-1} of NaCl treatment to 2.206 µmol g^{-1} at 100 mmol L^{-1}

Table 3. Proline content (µmol g^{-1}) in genipap (*Genipa americana* L., Rubiaceae) accessions subjected to NaCl concentrations in vitro, at 30, 60, and 90 days(1).

Accession(2)	Time (days)		
	30	60	90
	0 mmol L^{-1} NaCl		
NB	6.345Aa	4.875Bb	2.245Cb
CRA	2.225Cc	5.765Aa	3.780Ba
SA	2.745Cb	4.135Ac	3.515Ba
	25 mmol L^{-1} NaCl		
NB	2.665Bc	1.555Cb	4.395Aa
CRA	12.58Aa	4.895Ba	3.675Cb
SA	3.220Bb	4.410Aa	2.965Bc
	50 mmol L^{-1} NaCl		
NB	3.010Bb	2.975Bc	4.370Aa
CRA	2.685Bb	6.150Aa	2.575Bc
SA	6.790Aa	4.520Bb	3.090Cb
	75 mmol L^{-1} NaCl		
NB	3.110Cb	4.470Ab	4.030Aa
CRA	4.570Ba	6.100Aa	3.070Cb
SA	1.350Bc	4.300Ab	1.800Bc
	100 mmol L^{-1} NaCl		
NB	2.620Bb	4.030Aa	1.820Cb
CRA	2.580Ab	3.040Ab	2.03Bb
SA	6.620Aa	3.450Ca	4.260Ba
CV (%)		5.21	

(1)Means followed by equal letters, lower case in the columns, and upper case in the lines, do not differ, by the Tukey's test, at 5% probability. (2)Brazilian genipap accessions: NB, from Núcleo Bandeirante (Distrito Federal, DF); CRA, from Cruz das Almas (Bahia state); SA, from Sabinópolis (Minas Gerais state).

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NaCl. These differences among the accessions may be due to the influence of their genetic information, since proline would also be able to induce the expression of responsive genes induced by salt stress.

The increasing accumulation of proline showed a natural osmoregulation, since the accumulation allows of the plant physiological processes to continue, in addition to acting as osmoprotectors of macromolecules, such as proteins and lipids, replacing the water molecule in membranes due to the hydrophilic character. This accumulation promoted survival, although with lower biometric measurements than that of the treatment without NaCl.

The survival of plants exposed to abiotic stresses such as salinity may depend on osmotic adjustment mechanisms, which manifest themselves through the accumulation of inorganic ions or organic compounds. The proline accumulation promoted the survival and growth of explants from the SA, which is a fact related to osmotolerance. Proline can increase its value by up to 100-fold in plants subjected to stresses, in comparison with normal plants, and can be used as a marker for selecting salinity-tolerant individuals (Khan & Panda, 2008). The other accessions obtained lower concentrations of amino acid. Proline may be related to another type of response because, at low concentrations, it has a protective role (Cardoso et al., 2019). In genipap tree, studies involving the action of proline on seedling and on in vitro plant growth are still scarce; therefore, more research is necessary for a better understanding of the proline effects.

Conclusions

1. Germination and initial in vitro growth of genipap (*Genipa americana*) are affected by saline stress, with varying degrees of tolerance among accessions, for which the accession SA (from Sabinópolis) stood out.

2. The percentage of in vitro germination of genipap seed shows a positive linear reduction with the increase of NaCl in the culture medium.

3. The enzyme activities of superoxide dismutase and ascorbate peroxidase, in genipap seed subjected to saline stress in vitro, show the increase up to 288 hours of exposure.

4. The in vitro vegetative development of the accessions of genipap is affected by concentrations over 50 mmol L^{-1} NaCl.

5. There is greater accumulation of proline in the genipap leaves, in the presence of NaCl in the culture medium at 30, 60, and 90 days.

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The authors declare no conflicts of interest.

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