# Pretreatment of forage legumes under moderate salinity with exogenous salicylic acid or spermidine

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**ABSTRACT.** The present study aims to determine whether exogenous salicylic acid (SA) or spermidine (Spd) has any protective effect against salt stress. Seeds were subjected to 0, 20, 40, and 60 mM NaCl with or without salicylic acid or spermidine (0.5 mM) for 10 days. The evaluated variables were germination rate, shoot and root dry masses, glycine betaine content, lipid peroxidation, and the activities of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). The data were subjected to Tukey's test ($p \leq 0.05$). There was a growth increase, especially in plant shoots. The reduction in lipid peroxidation, as indicated by lower malondialdehyde (MDA) levels, can be explained by an increase in antioxidant activity when SA and Spd were added. When compared to CAT and APX, SOD was the least responsive enzyme to the addition of both SA and Spd in salt-stressed plants. SA and Spd partially reduced the effects of moderate salt stress in both plant species; however, Spd addition had better results than SA in terms of suppressing oxidative stress. Lablab plants were more vigorous than pigeonpea plants.

**Keywords:** *Cajanus cajan*; *Dolichos lablab*; salt stress; glycine betaine; oxidative stress; growth.

## Introduction

Saline soils are considered one of the greatest barriers to the growth and development of plants, representing one of the most worrying abiotic stresses for the scientific community. Pirasteh-Anosheh, Ranjbar, Pakniyat, and Emam, (2016) considered salinity to be most important abiotic stress and to be more important than drought because salt stress can occur anywhere, even when water resources are not limited. Typically, in salinated soils, sodium chloride (NaCl) constitutes the main salt (Munns, 2011). Plants that are not adapted to saline environments show many biochemical and physiological alterations under salt stress, and the study of these changes can aid crop breeding programs in selecting plants tolerant to this kind of stress (Cabello, Lodeyro, & Zurbriggen, 2014; Yousuf et al., 2017).

Many studies aim to verify the effects of salt stress on seed germination and seedling growth, as these are known to be the most sensitive phases in most plant species (Zapata, Serrano, Pretel, Amorós, & Botella, 2004; Munns & Tester, 2008; Park, Kim, & Yun, 2016) and thus can be used in salt tolerance screening (Latef & Ahmad, 2014; Rahman et al., 2017).

The effects of NaCl on plants include osmotic stress, due to lower cellular hydric potential and ionic stress due to the cytotoxicity of the saline ions $Na^+$ and $Cl^-$, leading to a nutritional imbalance mainly in K, Ca, Mg, and as a consequence of both stresses (osmotic and ionic), the production of reactive oxygen species (ROS), which mainly consist of singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$), superoxide anions ($O^{-2}$), and hydroxyl radicals (OH•), is triggered, which causes damage to living cells (Abogadallah, 2010; Munns & Gilliham, 2015; Pirasteh-Anosheh et al., 2016). In fact, at high concentrations, ROS (mainly $H_2O_2$) promote oxidative stress and trigger signaling events associated with cell death, but at low concentrations, ROS act as messenger molecules involved in adaptive signaling, allowing tolerance against various abiotic stresses (Abogadallah, 2010; Hao et al., 2012). According to Khan, Asgher, and Khan (2014), to counteract the

adverse effects of excess ROS production, plants induce mechanisms adapt to osmotic and ionic stresses that are caused by salt stress. Several studies have shown that the accumulation of the quaternary amine glycine betaine (GB) helps the plant system survive severe osmotic stress by acting as a "compatible solute" or "osmolyte", e., lowering the osmotic potential in the cytosolic compartment while not inhibiting metabolic reactions at relatively high concentrations (Fariduddin, Varshney, Yusuf, Ali, & Ahmad, 2013; Wani, Brajendra Singh, Haribhushan, & Iqbal Mir, 2013; Roychoudhury & Banerjee, 2016; Rahman et al., 2017).

In plants, oxidative stress by salinity increases the activity of the antioxidant enzymes superoxide dismutase (SOD - EC 1.15.1.1), catalase (CAT - EC 1.11.1.6), and ascorbate peroxidase (APX - EC 1.11.1.11), which are involved in the degradation of oxygenated active radicals, in the damage to proteins, and in the lipid peroxidation of cellular membranes (Sharma, Jha, Dubey, & Pessarakli, 2012; El-Beltagi & Mohamed, 2013; Park et al., 2016). Lipid peroxidation is one of the most harmful effects of oxidative stress because it causes fluidity of the cell membrane, ionic permeability alterations, and changes in other functions associated with membranes. Therefore, antioxidant enzyme activities and the MDA content often serve as key biochemical indicators to assess the sensitivity of plants to stress conditions (Sharma et al., 2012; El-Beltagi & Mohamed, 2013).

In the last few years, studies on the use of exogenously applied compounds as a tolerance strategy against the effects of many abiotic stresses have become relevant. Plant hormone antioxidants, signaling molecules, polyamines (PAs), and trace elements, among others, have been found to be effective in the mitigating salt-induced damage in plants (Hasanuzzaman, Nahar, Alam, Ahmad, & Fujita, 2015). As salicylic acid and spermidine are responsible for metabolic pathways that are interconnected with others involved in the formation of various relevant signaling molecules and metabolites in plants under stress responses, these substances have been studied as exogenous protectors in plants under salt stress. Some studies have reported that SA application can mitigate the impact of salt stress on plants by activation the antioxidant system, aiming to reduce oxidative stress losses and the leakage of membrane ions (Hayat, Hayat, Irfan, & Ahmad, 2010; Pál, Szalai, Kovács, Gondor, & Janda, 2013; Miura & Tada, 2014; Muthulakshmi & Lingakumar, 2017). Spermidine (Spd) is considered an important modulator involved in the regulation of plant growth and development, such as flower, leaf, and root differentiation, flower and fruit development, plant senescence, and seed and pollen germination (Kusano, Berberich, Tateda, & Takahashi, 2008). There is a considerable amount of literature on the role of PAs in protecting plants under stressful conditions (Gupta, Dey, & Gupta, 2013; Alcázar & Tiburcio, 2014; Sengupta, Chakraborty, Saha, Gupta, & Gupta, 2016).

With the assumption that SA and Spd can mitigate saline stress in plants, this study aimed to determine whether this outcome can also be observed in the seed germination and growth of young seedlings of two forage legumes. In addition, we also verified which of these exogenous metabolites has a higher mitigation potential against salinity.

## Material and methods

### Plant growth conditions

We evaluated two plant species: pigeonpea cv. BRS Mandarin and lablab bean cv. Rongai; two salt stress attenuators: salicylic acid and spermidine, at two concentrations (0.0 and 0.5 mM), and four concentrations (0, 20, 40, and 60 mM) of sodium chloride (NaCl) to induce different levels of salt stress. Each treatment had four replications of 25 seeds. The osmotic potentials of the NaCl ($\psi$s) solutions were 0.0, -0.089, -0.178, and -0.267, which were calculated according to the equation of Van't Hoff (Salisbury & Ross, 1992), as follows: $\psi = -i.C.R.T$; wherein: $\psi$ is the solute potential (MPa), $i$ is the isotonic coefficient (iNaCl = 1.8), $C$ is the solution molarity (mol of solute $kg^{-1}$ $H_2O$), $R$ is the gas constant (0.00831 MPa L $K^{-1}$ $mol^{-1}$), and $T$ is the absolute temperature (295.15 K).

### Seed germination and seedling growth

Seeds were sown in plastic boxes (11.0 x 11.0 x 3.5 cm) lined with two sheets of germination paper, moistened with 2.5 times the weight the of dry paper of solution and maintained in a greenhouse at a mean temperature of 25°C ± 1°C and a 12-hour photoperiod, determined by Rules for Seed Anal (BRASIL, 2009).

The germination standard was based on a primary root length of 2 mm with a positive geotropic curvature of the seeds (Duran & Tortosa, 1985). Ten days after sowing, the germination rate and seedling growth were measured by root and plumule dry mass measurements. For this procedure, seedlings, roots, and plumules were placed in a forced-air circulation oven at 65°C for 48 hours.

## Glycine betaine content

To measure the content of glycine betaine, dry and ground leaves were taken from plants grown under both normal and stressed conditions. The analysis was carried out according to the method of Grieve and Grattan (1983). The leaf extract was prepared in 50 mL test tubes by adding 0.5 g dry and ground leaves in 20 mL water. All the tubes were mechanically shaken at 25°C for 24 hours. After filtration, 1 mL extract was mixed with 1 mL 2-N $H_2SO_4$ solution, and then an aliquot of 250 µL was removed and kept in ice water for 1 hour. Afterwards, 0.1 mL potassium tri-iodide solution (containing 7.5 g iodine and 10 g potassium iodide in 100 mL 1-N HCl) was added and stirred for 5 seconds, and then the solution was covered with film paper to avoid volatilization. The tubes with the extracts were kept at 4°C for 16 hours to finish the reaction. Then, the tubes were centrifuged at 11,872 × $g$ for 15 minutes at 0°C. The supernatant was carefully removed leaving only periodate crystals, and then 4.5 mL 1,2-dichloroethane was poured into the solution. By passing a continuous stream of air into the solution for 2.30 hours, the two layers were separated, the upper aqueous layer was discarded, and the optical density of the organic layer was recorded at 365 nm. A standard curve of glycine betaine (50 - 200 µg mL$^{-1}$) was prepared in 1-N $H_2SO_4$. The results were expressed as mmol GB g$^{-1}$ dry mass.

## Lipid peroxidation

Lipid peroxidation was measured by estimating the content of thiobarbituric acid-reactive substances (TBARS), as described by Heath and Packer (1968). The extract was prepared using 0.25 ± 0.025 g ground leaf tissue with 20% polyvinylpyrrolidone (PVPP) (w/v) and 2 mL 0.1% trichloroacetic acid (TCA). After centrifugation at 11,000 × g for 15 min., the supernatant (250 µL) was added to 20% TCA (1 mL) along with 5% thiobarbituric acid (TBA) in a clean tube, mixed vigorously and incubated in a water bath at 95°C for 30 min. The reaction was stopped by cooling the tube in an ice bath for 10 min. and centrifuging at 12,000 × $g$ for 10 min. at 4°C. The concentration of malondialdehyde (MDA) equivalents was calculated using an extinction coefficient of $1.55 \times 10^{-5}$ mol$^{-1}$ cm$^{-1}$ with readings between 535 and 600 nm (Gratão et al., 2015), and the results were expressed as µmol MDA g$^{-1}$ fresh weight.

## Enzyme extraction and protein determination

Plumule samples were collected on the 10$^{th}$ day, placed in liquid $N_2$ and stored in a freezer at -80°C until the biochemical evaluations were performed. The gross enzyme extract was prepared by homogenizing the samples with 1 g plumules (3: 1 - v/v). the extraction buffer was composed of 100 mM potassium phosphate buffer (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 3 mM DL-dithiothreitol, and 5% insoluble polyvinylpolypyrrolidone (w/v) (Boaretto et al., 2014). The homogenate was centrifuged at 10,000 × $g$ for 30 min. and the supernatant was stored at -80°C for further determinations of superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) activities. The Protein concentration was determined following the method of Bradford (1976), using bovine serum albumin as a standard.

## Superoxide dismutase assay

SOD activity (EC 1.15.1.1) was determined as described by Giannopolitis and Ries (1977). An aliquot of 50 µL of plant extract was added to 1.45 mL reaction medium, which consisted of 50 mM sodium phosphate buffer (pH 7.8), 13 mM methionine, 63 µM nitro blue tetrazolium, 0.1 mM EDTA, and 1.3 µM riboflavin. The reaction was conducted at 25°C in a reaction chamber lit by a 15-W fluorescent bulb, and kept inside a closed box. After 5 minutes of exposure to light, illumination discontinued and the blue formazan produced by NBT photoreduction was measured at 560 nm. SOD activity was expressed as unit SOD mg$^{-1}$ protein.

## Catalase assay

Catalase activity (EC 1.11.1.6) was determined according to the method described by Kraus, Pauls, and Fletcher (1995), and modified by Azevedo, Alas, Smith, and Lea (1998). The reaction started by adding 25 µL

plant extract into a solution containing 1 mL 100-mM potassium phosphate buffer (pH 7.5) and 25 μL $H_2O_2$ (30% solution). The activity was determined by monitoring the decomposition of $H_2O_2$ at 240 nm over 1 min. CAT activity was expressed as μmol min.$^{-1}$ mg$^{-1}$ protein.

### Ascorbate peroxidase assay

Ascorbate peroxidase activity (EC 1.11.1.11) was tested by a reaction consisting of 80 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM EDTA, and 0.1 mM $H_2O_2$. It was measured by monitoring the rate of ascorbate oxidation at 290 nm and 30ºC, and was expressed as nmol ascorbate min.$^{-1}$ mg$^{-1}$ protein (Gratão et al., 2012).

### Statistical analysis

The experiment was analyzed in a completely randomized design, in a 2 x 3 x 4 factorial scheme. The statistical analyses were performed using AgroEstat software (Barbosa & Maldonado Júnior, 2010). A multiple comparison of means using Tukey's test was performed, followed by an individual ANOVA for each character at a 0.05 level of significance. The result for each plant was expressed the as mean and mean standard error (±SEM).

## Results and discussion

The alleviation of salinity stress using the exogenous application of several plant growth regulators such as SA and Spd has been of interest to researchers. In our study, we verified that only the SA application resulted in an increase in germination for both species under the lowest concentration of NaCl (20 mM) and that there was a decrease in germination under 60 mM NaCl for both species caused by the application of 0.5 mM SA and Spd when compared to the control. The application of a concentration of 0.5 mM SA to the seeds with or without salt stress had no significant effect on seed germination of either lablab or pigeonpea. However, Spd caused the greatest reduction in germination for both species, and this effect can be attributed to a reduction in pigeonpea seed germination compared to that of lablab, which was not affected by either of the compounds (Figure 1A and C). The NaCl concentrations (20 – 60 mM) used in this study did not affected the germination of lablab seeds and were not enough to cause water restriction and inhibit germination. However, the germination of pigeonpea seeds was reduced by all NaCl concentrations (Figure 1B), showing that salinity hampers seed germination by affecting the major events involved in germination such as imbibition, metabolism activation, embryonic tissue emergence, and seedling establishment (Rahman et al., 2017).

The level of PAs changes with salinity in most cases, with Spd levels increasing with salinity (Zapata et al., 2004). This increase could be one of the reasons for the lowest pigeonpea germination rates when using 0.5 mM Spd (Figure 1C). Moreover, exogenous Spd might have inhibited the stimulatory effects of plant hormones that participate in seed germination, such as gibberellins (GA) and ethylene (ET), or it could have stimulated abscisic acid (ABA), which is a powerful inhibitor of seed germination (Huang et al., 2017). The decrease in the germination rates of both species under salt stress caused by 0.5 mM SA and Spd might have occurred due to the effect of the low or slightly moderate NaCl concentrations used in this study (20 mM – 60 mM). The same results in lablab and in another pigeonpea cultivar (cv. Caqui) were found by Melloni et al. (2012), who verified that 0.5 mM Spd caused a significant decrease in seed germination under salt stress (20 – 120 mM). However, these authors showed that at this decrease was smaller than the one observed in treatments with no Spd, indicating that salt stress could mitigate the effect of this polyamine. For the exogenous SA, Dolatabadian. Modarres Sanavy, and Sharifi (2009) reported a mitigating effect at 0.5 mM SA on the germination of wheat seeds under harsher salt stress conditions (50 – 200 mM). The results found by the above mentioned authors reinforce our assumption that the lack of an increase in pigeonpea and lablab seed germinations when using exogenous SA and Spd is because of the NaCl concentrations used (20 – 60 mM), which might not have been enough to cause salt stress to make allow the compounds to act as attenuators (Figure 1B).

Indeed, although some studies have implied that SA has a vital function in salt stress mitigation in seed germination for many plant species, others have reported that the seed germination responses to SA use under several abiotic stresses are ambiguous, as evidenced by conflicting results, i.e., being able to inhibit germination or increase seed vitality; therefore, the cause of these results may rely on the concentration

used (Hayat et al., 2010; Rivas-San Vicente & Plasencia, 2011; Tan, Chen, Wang, & Dai, 2013; Jayakannan, Bose, Babourina, Rengel, & Shabala, 2015). Studies conducted by Lee et al. (2010) demonstrated that high SA concentrations (> 100 μM) inhibit the germination of Arabidopsis seeds under salt stress, whereas low SA concentrations (> 50 μM) mitigate the salt stress effects; thus, the effects of SA on seed germination are dose-dependent. In fact, the role of SA may vary with the stress level, i.e., moderate or severe, even if there is an interaction between the downstream signals of ROS and SA, in which redox regulation plays a key role (Barba-Espín et al., 2011). Nevertheless, the concentration of 0.5 mM SA or Spd might have altered the ratio of gibberellin (GA) to abscisic acid (ABA) in the pigeonpea and lab lab under saline stress; these are hormones that modulate seed germination, so when GA is suppressed, germination is stimulated, or when ABA is induced, germination is inhibited (Rajjou et al., 2006; Tan et al., 2013; Huang et al., 2017). The germination inhibition by exogenous SA could be based on oxidative stress. Some studies have shown that the effect of SA as a negative regulator of seed germination might be due to SA-induced oxidative stress, especially because of $H_2O_2$ accumulation (Lee et al., 2010; Gill & Tuteja, 2010; Rivas-San Vicente & Plasencia, 2011; Park et al., 2016). Additionally, studying the effects of SA on seed germination in *Arabidopsis* mutants under salt stress, Hao et al. (2012) stated that the role of this substance in plants under salt stress is controversial since exogenous SA associated with endogenous SA can lead to the accumulation of SA, which negatively interferes with the mitigation of salinity effects. This process could be one of the explanations for the inhibition of germination caused by exogenous SA that occurred in the species under salt stress in this study (Figure 1A and C).
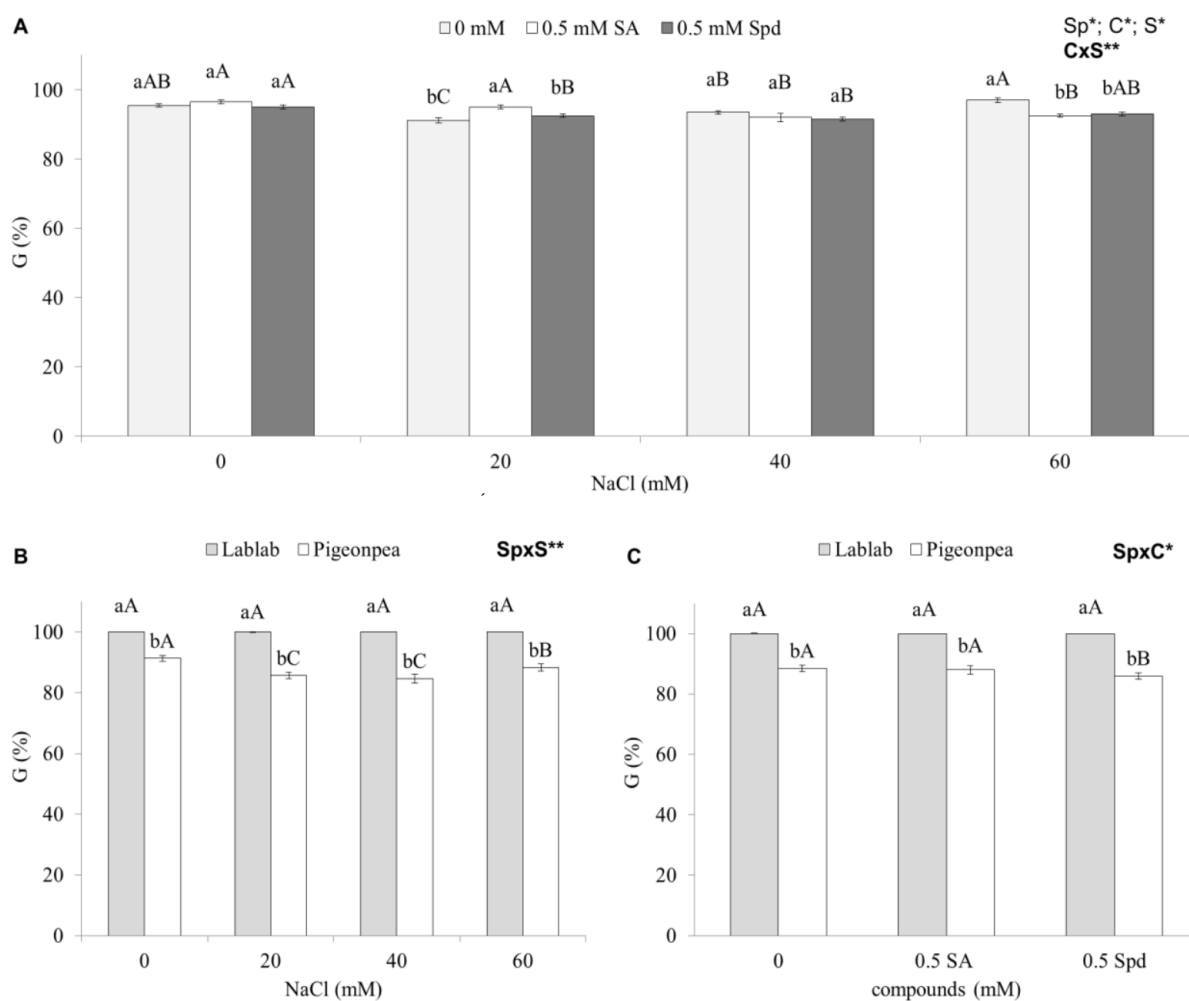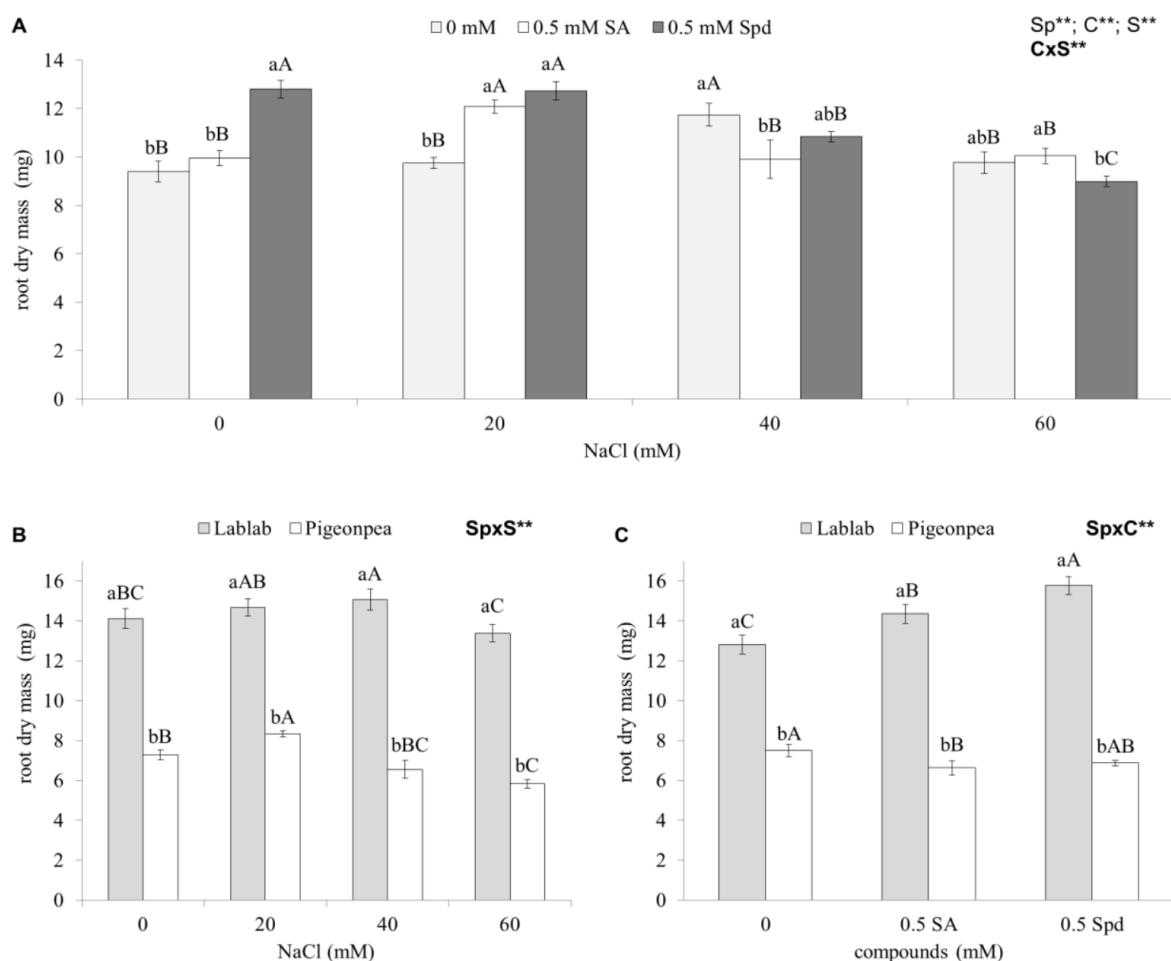


**Figure 1.** Average rates of germination of pigeonpea cv. BRS Mandarin (G) and lablab cv. Rongai at 10 days after sowing. A – Effect of salicylic acid (SA) and spermidine polyamine (Spd). B – Effect of NaCl concentrations. C – Interaction between compounds (SA and Spd) and salinity (NaCl). Averages followed by the same lowercase letter (within concentrations) or capital letter (between concentrations) do not differ from each other by Turkey's test (p > 0.05). Averages of four replicates. Species (Sp), compounds (C), and salinity (S). **significant at 1% probability by the F-test, *significant at 5% probability by the F-test, and vertical bars show standard errors (±SE).

Not surprisingly, salt stress damages many plant species during the seedling and early developmental stages; however, other stages are the most susceptible to salinity during the entire life cycle of plants (Rahman et al., 2017). The decline in cellular growth processes is due to dehydration resulting from the osmotic effect of salts accumulating in the root zone and due to the toxic effect of sodium and chloride accumulating in plant tissues, which greatly damage the most important physiological processes and cell membrane integrity (Munns & Tester, 2008). Our findings demonstrated that there was an increase in shoot growth in pigeonpea and lablab seedlings with the use of SA and Spd when under severe saline stress at 40 and 60 mM NaCl (Figure 2A). Similarly, Fariduddin Khan, Yusuf, Aafaqee, and Khalil (2017) showed that salt-stressed plants treated with SA or Spd increased in dry mass when compared to those grown untreated. However, these compounds had no effect on the growth of the plumules of pigeonpea but stimulated a significant increase in plumule growth for lablab seedlings (Figure 2C). In short, positive effects of using SA and Spd were only observed on the initial shoot growth of lablab seedlings (Figure 2A and C). Moreover, the results also highlighted that in the absence of salt stress (0.0 mM NaCl), both SA and Spd increased shoot growth in lablab and pigeonpea (Figure 2A). Therefore, SA and Spd seem to induce the growth of pigeonpea and lablab plumules, whether under salt stress or not. At 20 mM NaCl, SA reduced shoot growth in both species compared to that of the treatments with or without Spd (Figure 2A). At that salt concentration, there was also a reduction in shoot growth in the lablab seedlings compared to that in the pigeonpea seedlings (Figure 2B), which could have contributed to the lower growth caused by SA at 20 mM (Figure 2A) since the concentrations used in this study (20 – 60 mM NaCl) caused no salinity effect on the growth of pigeonpea plumules (Figure 2B).
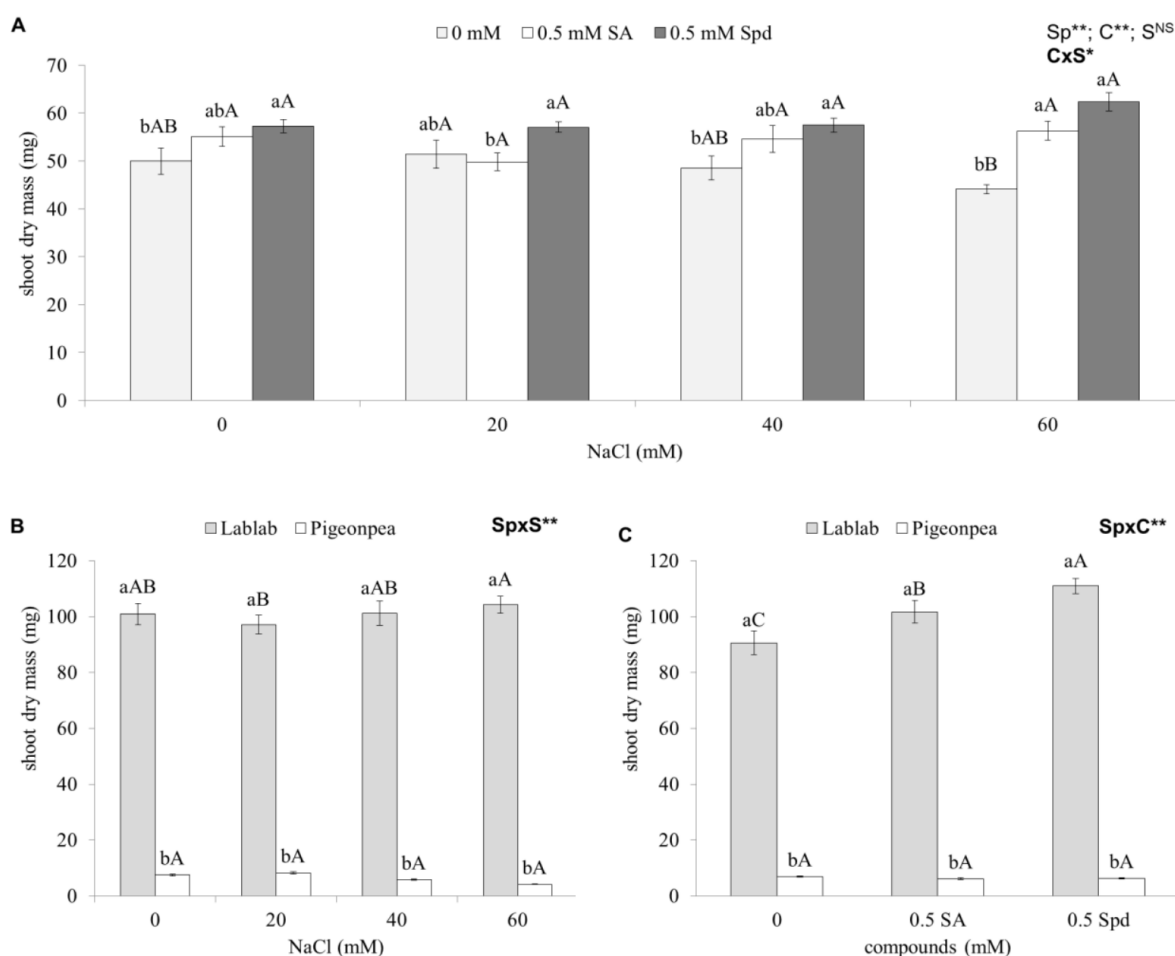


**Figure 2.** Average shoot dry mass of pigeonpea cv. BRS Mandarin (SDM) and lablab cv. Rongai at 10 days after sowing. A – Effect of salicylic acid (SA) and spermidine polyamine (Spd). B – Effect of NaCl concentrations. C – Interaction between compounds (SA and Spd) and salinity (NaCl). Averages followed by the same lowercase letter (within concentrations) or capital letter (between concentrations) do not differ from each other by Turkey's test (p > 0.05). Averages of four replicates. Species (Sp), compounds (C), and salinity (S). **significant at 1% probability by the F-test, *significant at 5% probability by the F-test, and vertical bars show standard errors (±SE).

Compared to the absence of SA an Spd, the presence of both SA and Spd increased growth in pigeonpea and lablab roots when the NaCl concentrations were 0.0 mM and 20 mM (Figure 3A). In both studied species, the concentration of 20 mM NaCl had did not have a negative impact on root growth. Conversely, the concentrations of 40 and 60 mM NaCl decreased root growth when using both compounds, with the greatest reduction at 60 mM NaCl (Figure 3A). This NaCl concentration was the most harmful for both species in terms of root growth (Figure 3B), while the other salt concentrations did not have a negative effect on this variable. Moreover, when compared to the control (0.0 mM NaCl), the concentrations of 40 and 60 mM NaCl with the addition of SA or Spd promoted root growth in pigeonpea and lablab (Figure 3B). Lablab root growth was increased by the use of 0.5 mM SA or Spd, whereas both compounds reduced root growth in pigeonpea (Figure 3C). Studying two other pigeonpea cultivars (IAC Fava Larga and Caqui), Destro, Santos, Vollet, Marin, and Banzatto (2008) reported a reduction in root growth when the plants were under salt stress and received 0.5 mM exogenous Spd.



**Figure 3.** Average root dry mass of pigeonpea cv. BRS Mandarin (RDM) and lablab cv. Rongai at 10 days after sowing. A – Effect of salicylic acid (SA) and spermidine polyamine (Spd). B – Effect of NaCl concentrations. C – Interaction between compounds (SA and Spd) and salinity (NaCl). Averages followed by the same lowercase letter (within concentrations) or capital letter (between concentrations) do not differ from each other by Turkey's test ($p > 0.05$). Averages of four replicates. Species (Sp), compounds (C), and salinity (S). **significant at 1% probability by the F-test, *significant at 5% probability by the F-test, and vertical bars show standard errors (±SE).

Among the compatible solutes, GB is a particularly effective osmolyte against abiotic stress, alleviating the negative effects of salt stress in several species (Chen & Murata, 2011). However, our findings showed that, in both species, there was a significant decrease in GB with increasing NaCl concentrations (Figure 4B). Although many studies have related GB accumulation to plants that are tolerant to salt stress in comparison with that of sensitive plants (Liang et al., 2009 Fariduddin et al., 2013; Roychoudhury & Banerjee, 2016; Tian et al., 2017), not all plants under abiotic stress accumulate GB (Giri, 2011; Wani et al., 2013). In this particular case, it cannot be said that pigeonpea and lablab belong to the category of GB non-accumulating

plants in response to salt stress, as this effect is not only species-specific, but also depends on cultivars within the same species (Giri, 2011; Wani et al., 2013). For example, some genotypes of sorghum and corn accumulate GB, whereas others do not (Kurepin et al., 2015). Furthermore, in this study, the presence of 0.5 mM SA or Spd did not promote GB accumulation to suppress salt stress in the seedlings of both species. Instead, these compounds drastically reduced the levels of GB (Figure 4A and C). It already has been reported that SA is one of the GB biosynthesis in barley under salt stress (Jagendorf & Takabe, 2001; Horváth, Szalai, & Janda, 2007). Similarly, the influence of applying 0.5 mM SA on the alleviation of salt stress (100 mM) by increasing GB accumulation has already been shown in lentils (Misra & Saxena, 2009) and mungbean (Khan, Asgher & Khan, 2014). As an SA-mediated increase in GB levels can improve overall plant growth (Khan, Fatma, Per, Anjum, & Khan, 2015), exogenous SA was expected to promote the accumulation of this osmolyte in pigeonpea and lablab to suppress salt stress. Exogenous PAs have been documented to reduce salinity stress-induced damage, including the accumulation of compatible osmolytes (Saleethong, Roytrakul, Kong-Ngern, & Theerakulpisut, 2016). In contrast, our findings showed that exogenous Spd decreased GB accumulation (Figure 4). Even though our results on germination showed that lablab seedlings were less affected by salt stress and exogenous Spd (Figure 1A, B, and C), Spd caused a greater decrease in GB in lablab plants than in pigeonpea plants (Figure 4C). Destro et al. (2008) showed that in two genotypes of pigeonpea, namely the cultivars IAC Fava Larga and Caqui, which were grown in a hydroponic system for 20 days under salt stress (20 – 80 mM) and the presence of 0.5 mM Spd, a significant decrease in GB accumulation was observed at the concentration of 80 mM NaCl for 'IAC Fava Larga' and at 60 and 80 mM NaCl for 'Caqui'. Under salt stress, some plants increase the activity of betaine aldehyde dehydrogenases (BADH), which is widely considered a key enzyme in GB metabolism in higher plants (Liang et al., 2009; Wani et al., 2013). The decrease in GB in lablab and pigeonpea seedlings with 0.5 mM SA and exogenous Spd under salt stress is possibly related to the inhibition of BADH activity in GB biosynthesis.
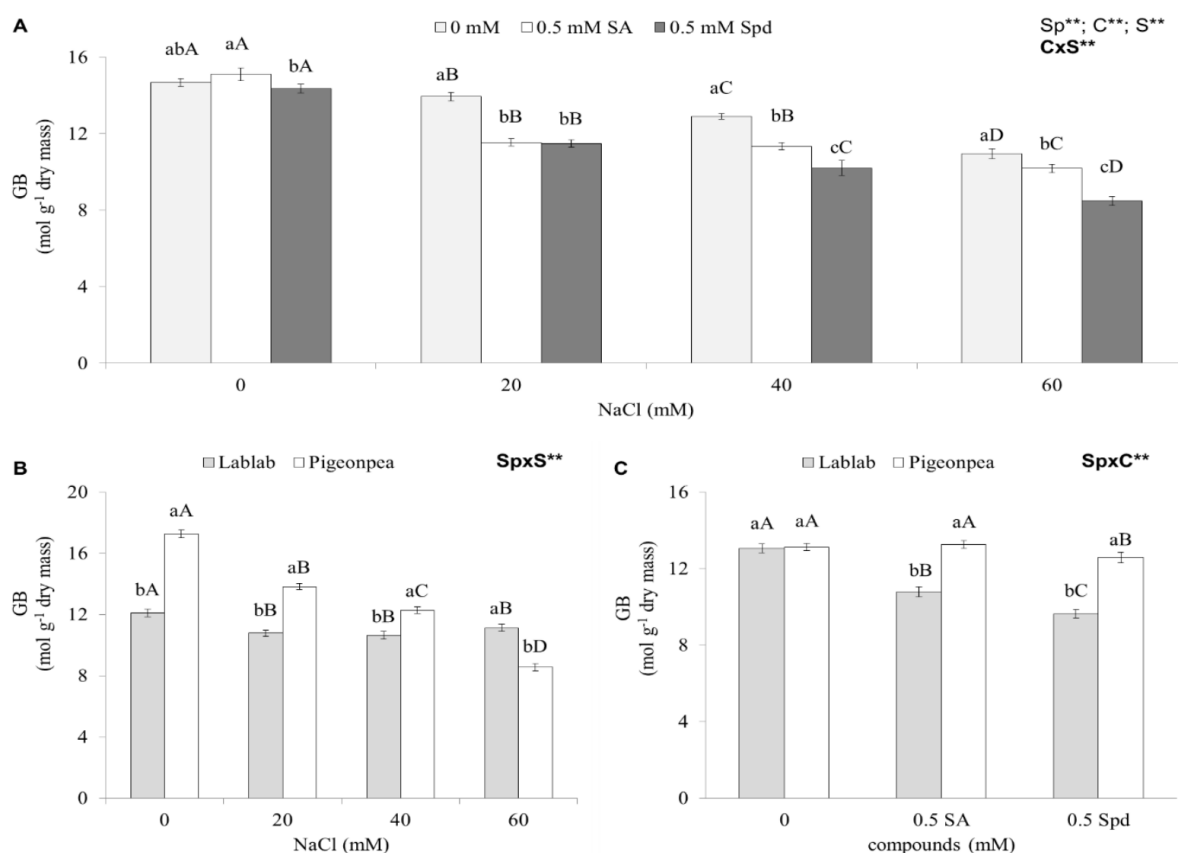


**Figure 4.** Average values of betaine glycine (GB) levels in pigeonpea cv. BRS Mandarin and lablab cv. Rongai at 10 days after sowing. A – Effect of salicylic acid (SA) and spermidine polyamine (Spd). B – Effect of NaCl concentrations. C – Interaction between compounds (SA and Spd) and salinity (NaCl). Averages followed by the same lowercase letter (within concentrations) or capital letter (between concentrations) do not differ from each other by Turkey's test (p > 0.05). Averages of four replicates. Species (Sp), compounds (C), and salinity (S). **significant at 1% probability by the F-test, *significant at 5% probability by the F-test, and vertical bars show standard errors (±SE).

Because salinity stress enhances the level of free radicals in plants, membrane damage was investigated by monitoring the MDA content (Figure 5). The MDA level in plants has been widely used as an indicator of salt-initiated lipid peroxidation in membranes (Sharma et al., 2012; El-Beltagi & Mohamed, 2013). Our results on the MDA content showed that the harmful effects of salt stress on the integrity of cell membranes were mitigated by applying 0.5 mM SA to both studied species, in contrast with that of the untreated plants (Figure 5A). SA application reduced lipid peroxidation at all NaCl concentrations (Figure 5A). Dolatabadian et al. (2009) verified that the MDA content was reduced in wheat seedlings under salt stress treated with 0.5 mM SA. Pál et al. (2013) observed a decrease in lipid peroxidetion with the use of exogenous SA. According to Hayat, Hayat, Alyemeni, and Ahmad (2013), SA can delay membrane deterioration due to lipid peroxidation, which is known to be one of the adverse effects of salt stress, leading to MDA accumulation. In contrast, exogenous Spd had no effect on the MDA content, which was statistically similar between the treatments and the control (0.0 mM NaCl) (Figure 5A). When seedlings were grown under 0.0 and 20 mM NaCl, the MDA content was reduced by Spd in comparison with that by SA (Figure 5A). At 40 and 60 mM NaCl, neither the presence or absence of the compound showed significant differences in MDA content (Figure 5A). At all NaCl concentrations tested, the MDA levels in pigeonpea plants were significantly lower than those of lablab plants (Figure 5B). As salt stress was increased, lablab seedlings had lower contents of MDA (Figure 5B), which might have occurred due to the presence of Spd, which could have been responsible for reducing the MDA contents in this species (Figure 5C). In rice plants, an exogenous pretreatment of Spd (1.0 mM) prevented lipid peroxidation, and decreased $H_2O_2$ accumulation and electrolyte leakage by stabilizing the membrane (Roychoudhury, Basu, & Sengupta, 2011; Saleethong, Sanitchon, Kong-Ngern, & Theerakulpisut, 2011). However, in the case of pigeonpea, the decrease in the MDA content observed for seedlings treated with 60 mM NaCl (Figure 5B) had no relationship with the presence of 0.5 mM SA or Spd because no response was observed with the absence of these compounds (Figure 5C).
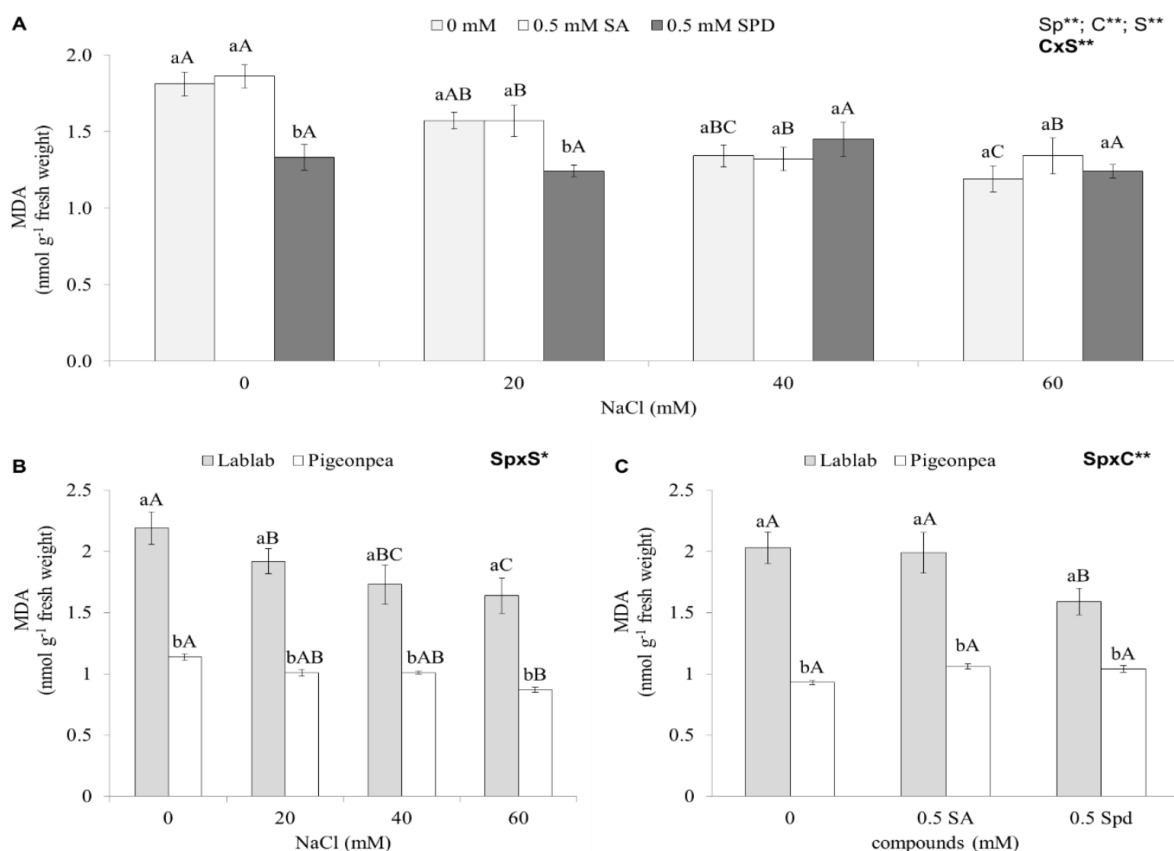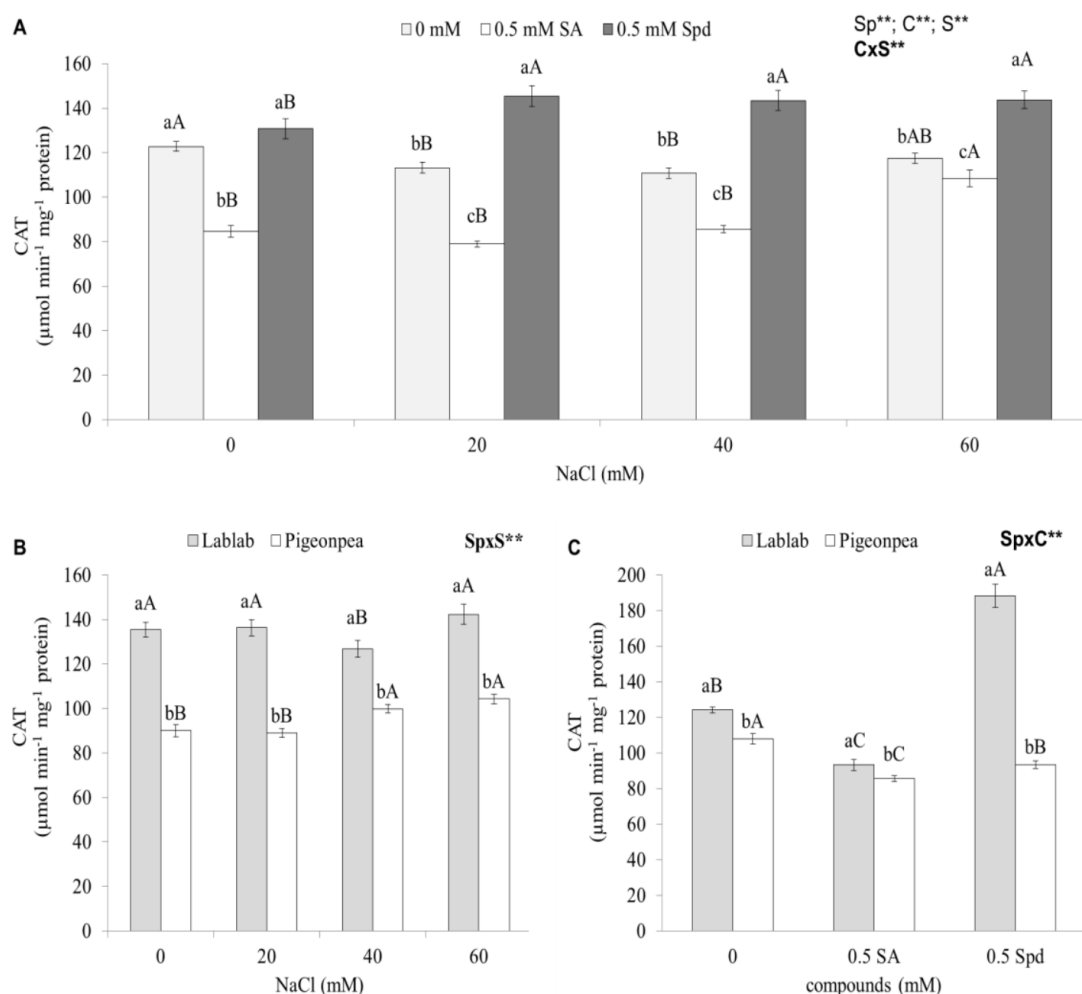


**Figure 5.** Average values of malondialdehyde (MDA) levels in pigeonpea cv. BRS Mandarin and lablab cv. Rongai at 10 days after sowing. A – Effect of salicylic acid (SA) and spermidine polyamine (Spd). B – Effect of NaCl concentrations. C – Interaction between compounds (SA and Spd) and salinity (NaCl). Averages followed by the same lowercase letter (within concentrations) or capital letter (between concentrations) do not differ from each other by the Turkey's test (p > 0.05). Averages of four replicates. Species (Sp), compounds (C), and salinity (S). **significant at 1% probability by the F-test, *significant at 5% probability by the F-test, and vertical bars show standard errors (±SE).

ROS-detoxifying antioxidant enzymes (SOD, CAT, and APX) are an effective defense mechanism against oxidative damage in plants. For both species, compared with Spd supplementation, SA supplementation at each saline concentration caused a severe reduction in SOD and CAT activities (Figures 6A and 7A, respectively). This decrease seems to have no relation to salinity since the use of SA in the control treatments (0.0 mM NaCl) also reduced the activity of both enzymes (Figures 6A and 7A, respectively). The presence of Spd increased SOD activity only at 20 mM NaCl in comparison with its absence (Figure 6A). Moreover, Spd increased CAT activity in plants under each saline concentration (20 mM, 40 mM, and 60 mM) in comparison with its absence (Figure 7A). In seedlings of both species without SA or Spd addition, the SOD activity increased at 40 and 60 mM NaCl when compared to that at 0.0 and 20 mM NaCl (Figure 6A); however, CAT activity was reduced at 20 and 40 mM NaCl (Figure 7A). SOD and CAT activities were always higher in lablab than in pigeonpea seedlings, whether they were under salt stress or not (Figures 6B and 7B, respectively) and with or without SA or Spd addition (Figures 6C and 7C, respectively). However, salinity did not increase the SOD activity in the lablab seedlings at any NaCl concentration compared with the control contrast, the SOD activity was reduced at the concentration of 60 mM (Figure 6B). The same was true for the CAT results, in which its activity was reduced at 40 mM NaCl (Figure 7B). When verifying the salt stress effects on SOD and CAT in pigeonpea plants, we noted that the concentrations of 40 and 60 mM NaCl significantly increased the activity of both enzymes (Figures 6B and 7B, respectively). However in lablab, the addition of 0.5 mM SA promoted a reduction in SOD and CAT activities compared to the control with the addition of Spd (Figures 6C and 7C, respectively). Moreover, CAT activity was higher with 0.5 mM Spd than with no Spd (Figure 7C), which was not observed for SOD (Figure 6C). Similarly, Duan, Li, Guo, and Kang (2008) reported that CAT activity increased with the addition of 0.01 mM Spd in cucumber under 50 mM NaCl stress, so they concluded that Spd can be easily used to improve the establishment of cucumber crops directly sown in a saline medium.
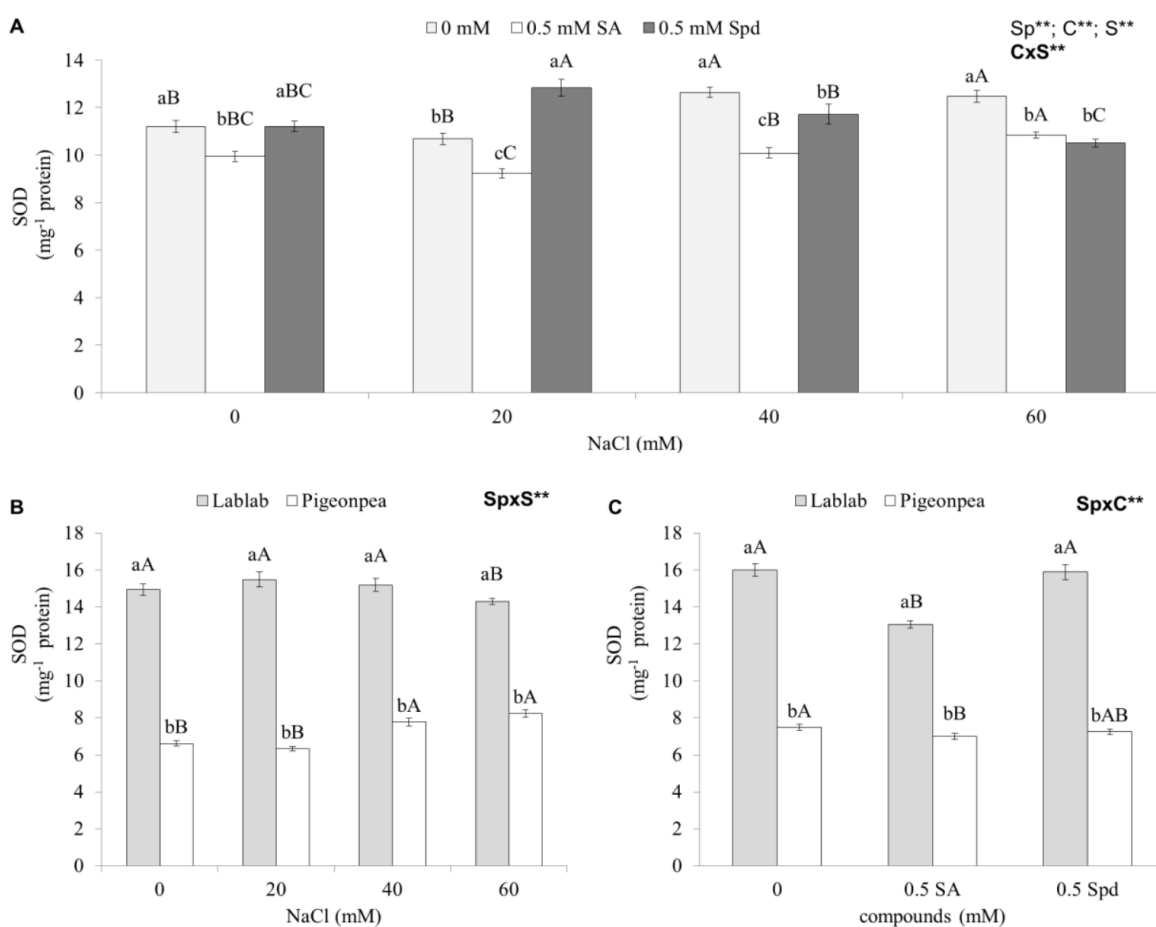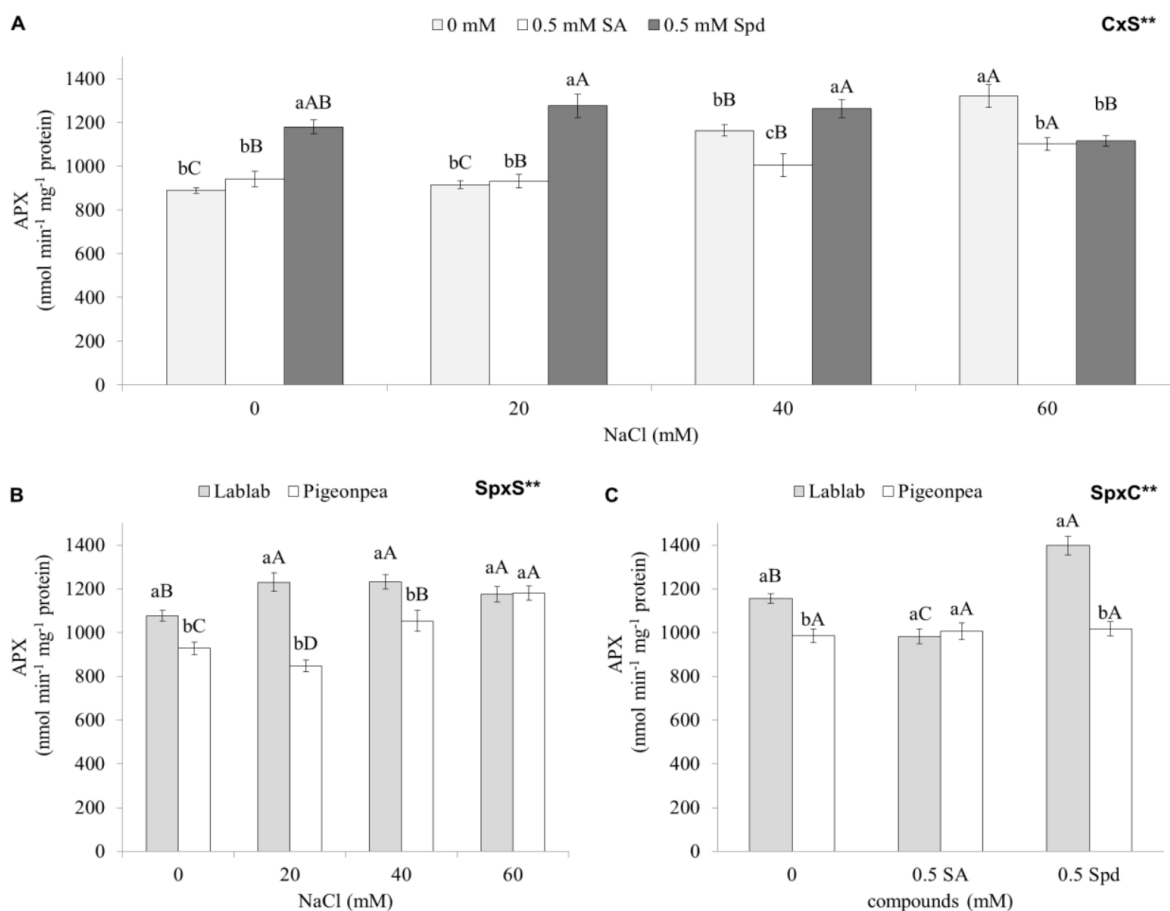


**Figure 6.** Superoxide dismutase (SOD) specific activity in pigeonpea cv. BRS Mandarin and lablab cv. Rongai at 10 days after sowing. A – Effect of salicylic acid (SA) and spermidine polyamine (Spd). B – Effect of NaCl concentrations. C – Interaction between compounds (SA and Spd) and salinity (NaCl). Averages followed by the same lowercase letter (within concentrations) or capital letter (between concentrations) do not differ from each other by Turkey's test (p > 0.05). Averages of four repetitions. Species (Sp), compounds (C), and salinity (S). **significant at 1% probability by the F-test, *significant at 5% probability by the F-test, and vertical bars show standard errors (±SE).

With respect to APX activity, Spd addition had a significant effect at all saline concentrations except for 60 mM in comparison with the absence of Spd and presence of SA (Figure 8A). In both species, none of the saline concentrations increased APX activity with the addition of SA (Figure 8A); indeed, a sharp drop in APX activity was observed at 40 mM NaCl compared to that with the absence of SA absence or Spd addition (Figure 8A). Additionally, in both species, the saline treatment of 60 mM significantly increased APX activity, which was not observed when the compounds were added (Figure 8A). At saline concentrations of 40 and 60 mM, the addition of Spd promoted increases in both APX (Figure 8A) and CAT (Figure 7A) activities; however at 60 mM, APX activity was reduced, while CAT activity increased (Figure 7A and 8A, respectively). At the same NaCl concentration (60 mM), SA addition increased the activities of the enzymes APX (Figure 8A), SOD (Figure 6A), and CAT (Figure 7A) for both plant species. Conversely, the absence of SA or Spd caused an increase in the activities of APX (Figure 8A) and SOD (Figure 6A) in both species. Through several studies, Rahman et al. (2017) verified that exogenous Spd (1 mM) conferred salt stress tolerance to different rice cultivars by improving the activity of the antioxidant enzymes CAT and APX. In contrast to the results obtained for SOD and CAT (Figures 6B and 7B, respectively), APX activity was similar for both pigeonpea and lablab seedlings (Figure 8B). Nevertheless, this response could be the result of the effect of 60 mM NaCl, which decreased APX activity in lablab but increased APX activity in pigeonpea. Therefore, NaCl concentrations above 60 mM could actually cause different responses in lablab and pigeonpea plants.



**Figure 7.** Catalase (CAT) specific activity in pigeonpea cv. BRS Mandarin and lablab cv. Rongai at 10 days after sowing. A – Effect of salicylic acid (SA) and spermidine polyamine (Spd). B – Effect of NaCl concentrations. C – Interaction between compounds (SA and Spd) and salinity (NaCl). Averages followed by the same lowercase letter (within concentrations) or capital letter (between concentrations) do not differ from each other by Turkey's test (p > 0.05). Averages of four repetitions. Species (Sp), compounds (C), and salinity (S). **significant at 1% probability by the F-test, *significant at 5% probability by the F-test, and vertical bars show standard errors (±SE).

Furthermore, the NaCl concentrations used here increased the activity of APX in lablab when compared to the APX activity under the control (Figure 8B). Unlike the lablab results for SOD and CAT (Figures 6B and 7B, respectively), APX activity was more responsive to the studied salt stress (Figure 8B). However, genetic differences in salt tolerance have been suggested to not be related to variations in the ability of plants to detoxify ROS; this is because *Arabidopsis* mutants lacking cytosolic and/or chloroplastic APX appeared to be more salt tolerant than the wild-type plants (Abogadallah, 2010). In pigeonpea, APX activity was higher at 60 mM NaCl than at 40 mM (Figure 8B). Both concentrations promoted a higher APX activity when compared to that at 20 and 0.0 mM NaCl (Figure 8B). While SOD and CAT showed a higher activity at 40 mM and higher (Figures 6B and 7B, respectively), for APX, the increase was seen at the concentration of 60 mM NaCl (Figure 8B). When comparing the addition of SA or Spd in pigeonpea and lablab (Figure 8C), SA showed no differences in terms of APX activity for both species (Figure 8C). In both lablab and pigeonpea, SOD and CAT showed increased activity, whether in the absence or presence of SA (Figures 6A and 7A, respectively). The APX response might have been because SA caused a large reduction in APX activity reduction in lablab plants in comparison with its absence (Figure 8C). Such an outcome was not observed in pigeonpea, which showed the same APX activity with or without SA (Figure 8C). In fact, the presence of SA or Spd caused no changes in APX activity for pigeonpea. For lablab, in contrast to the SA reducing effect, the addition of Spd increased APX activity significantly in comparison with its absence (Figure 8C). According to Abogadallah (2010), although the activities of antioxidant enzymes have been intensively studied, their importance in salt tolerance is still controversial because a high antioxidant enzyme activities have been associated with both salt tolerance and sensitivity.



**Figure 8.** Peroxidase ascorbate (APX) specific activity in pigeonpea cv. BRS Mandarin and lablab cv. Rongai at 10 days after sowing. A – Effect of salicylic acid (SA) and spermidine polyamine (Spd). B – Effect of NaCl concentrations. C – Interaction between compounds (SA and Spd) and salinity (NaCl). Averages followed by the same lowercase letter (within concentrations) or capital letter (between concentrations) do not differ from each other by Turkey's test (p > 0.05). Averages of four replicates. Species (Sp), compounds (C), and salinity (S). **significant at 1% probability by the F-test, *significant at 5% probability by the F-test, and vertical bars show standard errors (±SE).

# Conclusion

The addition of SA or Spd did not mitigate the effects of moderate salt stress on seed germination of either pigeonpea or lablab. There was a growth increase, especially of the plant shoot with addition of SA and Spd. The reduction in lipid peroxidation, indicated by lower MDA levels, can be explained by an increase in the activities of SOD and APX when adding SA and by an increase in the activity CAT when adding Spd. SOD was the least active enzyme in salt-stressed plants with the addition of both SA and Spd. SA and Spd partially reduced the effects of moderate salt stress in both plant species; however, the addition of Spd had better results than SA on suppressing oxidative stress. Lablab plants were more vigorous than pigeonpea plants. The information concerning plants subjected to NaCl and the addition of attenuators should provide a better understanding of the mechanisms of detoxification, which may help units biochemical genetics with plant breeding to develop tolerant plants.

# Acknowledgements

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