Agro Bali : Agricultural Journal
Vol. 7 No. 3: 800-809, November 2024

e-ISSN 2655-853X
https://doi.org/10.37637/ab.v7i3.1948

Salinity Stress and Exogenous Ascorbic Acid: Impact on Soybean Physiological Attributes and Biomass Accumulation

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Article history: submitted: July 28, 2024; accepted: November 4, 2024; available online: November 26, 2024

Abstract. Salinity, prevalent abiotic stress, has substantially restricted soybean production worldwide. This intricate environmental factor disrupts various physiological and biochemistry processes in soybean plants and ultimately diminishes yields. This experiment was conducted to report the damaging effect of sodium chloride (NaCl) given to the soil through watering at different levels (0, 50, and 100 mM NaCl) and to explore the impact of foliar spraying ascorbic acid to reduce the adverse effects of salinity at different levels (0, 400, 600, and 800 ppm). This study showed that the impact of salinity level significantly reduced gas exchange parameters, number of stomata, content of AsA, shoot dry weight, and root dry weight. The salinity also caused an increase in electrolyte leakage. Foliar application of ascorbic acid alleviated salinity-induced plant stress by increasing the number of stomata and root dry weight.

Keywords: biomass; exogenous ascorbic acid; physiological traits; salinity; stress mitigation

INTRODUCTION

Salinity is an important issue that should be a major concern because it potentially threatens food security. Soil salinity in agricultural land can increase due to fluctuations in weather, such as excessive or insufficient rainfall that causes salt to rise to the surface and seawater intrusion (Corwin, 2021). In Indonesia, the potential for land prone to salinization reaches 12.02 million hectares or 6.20% of Indonesia's total land area (Karolinoerita & Yusuf, 2020). Salinity can affect plant growth and yields and hinder sustainable agricultural development. The inhibitory effects of salinity on plants include causing osmotic stress and ion toxicity resulting from an increase in Na^+ and Cl-ions, as well as increasing the production of free radicals in plants.

Plants subjected to salinity stress typically exhibit diverse adaptive responses contingent upon the plant species, growth stage, salinity level, and other environmental parameters. Plants growing in saline conditions activate various mechanisms that help plants reduce stress's effects. Alterations in morphological, physiological, and biochemical properties, including increased antioxidant activity, indicate plant adaptation responses (El Sabagh et al., 2020).

Sodium chloride (NaCl) is the main salt contained in saline soil (Karolinoerita & Yusuf, 2020). The accumulation of high NaCl content causes plants to absorb more Na^+ and Cl^- ions. These ions are absorbed by root cells and transported through the xylem to the plant canopy (Wani et al., 2020). Excessive absorption of Na^+ and Cl^- ions can result in a nutritional imbalance in plants, where the absorption of K^+ ions is reduced, thereby causing metabolic, biochemical, and physiological processes in the plant body to be disrupted (Noreen et al., 2021). In this case, salinity negatively affects plant physiology, disrupting growth and decreasing biomass.

Soybean is a strategically important food crop commodity in Indonesia. As a primary source of vegetable protein, soybean seeds are essential food ingredients. Moreover, soybeans can be processed into a variety of products such as tempeh, tofu, milk, and soy sauce. However, salinity stress adversely affects soybean growth, development and yield components (Le et al., 2021). The strategy for increasing salinity stress tolerance on plants is by adding antioxidants. Antioxidants are known to increase osmoprotectant activity and have a positive effect on the physiology, biochemistry, growth, and yield of plants under salinity stress (Selem et al. 2022). Ascorbic acid is an

e-ISSN 2655-853X
https://doi.org/10.37637/ab.v7i3.1948

antioxidant that has an important role when plants face environmental stress and it is produced in the plant body. However, exogenous ascorbic acid is required when the endogenous ascorbic acid content is not sufficient to alleviate stress.

Tolerance in plants to salt stress can help maintain plant resilience, where plants can still maintain yield quality and reduce losses even though they grow under salt stress. However, the effectiveness of ascorbic acid varies greatly, depending on the stage of plant growth, method and interval of application, genetics of the plant species, and type of stress (Akram et al. 2017). Therefore, the current study's objectives were to examine the effect of foliar spraying of ascorbic acid on soybeans under salinity stress. The changes in physiological traits and biomass were monitored in response to NaCl and ascorbic acid treatments.

METHODS

The research was carried out from October 2023 to January 2024 in a screen house in Surakarta City, Central Java, at 96 meters above mean sea level. The materials used in this research were soybean Anjasmoro variety, Ascorbic Acid pro-analysis, and NaCl pro-analysis. The chemical and physical soil analysis results are presented in **Table 1**. The experiment employed a factorial completely randomized design (CRD) consisting of two factors with three replicates. The first factor is the NaCl concentration level (0, 50, 100 mM), and the second is the ascorbic acid (AsA) concentration level (0, 400, 600, 800 ppm). NaCl application was carried out using the method of Ghorbanli et al. (2004), by adding 25 mM every 2-day interval through watering until the target treatment concentration was reached. AsA is applied through the leaves at once-a-week intervals.

Table 1. Assessment of the experimental soil

Soil Properties	Value
pH	6.1
C-Organik	1.76%
N-Total	0.26%
C/N	6.8
P_2O_5 HCl 25%	71 mg.100gram^{-1}
K_2O HCl 25%	22 mg.100gram^{-1}
Available P	136 mg.kg^{-1}
Sand	65%
Silt	33%
Clay	2%
Electrical conductivity	0.74 dS.m^{-1}

The rate of photosynthesis (A), transpiration (E), and stomatal conductance (*gs*) were determined on fully opened leaves (second or third leaves) with the NY-1020 Plant Photosynthesis Meter. A replica method was employed to determine the number of stomata, and replicas were then viewed under a microscope at 40x magnification for stomata counting. AsA content was measured using the iodometric titration method. A 10-gram leaf sample was crushed and homogenized in 100 ml of distilled water. The mixture was filtered, and then 25 ml of filtrate was taken, and 2 ml of 1% amylum was added as an indicator. The solution was titrated with a 0.01 N iodine solution until it turned dark blue. The vitamin C concentration was then calculated based on the volume of iodine solution used. Electrolyte leakage was determined following the procedure described by Dionisio-Sese and Tobita (1998), fresh leaves (0.5 grams) were combined with 15 ml of double-distilled water in a test tube. The samples were heated to 40˚C for 60 minutes, and then the extract's first electrical conductivity (EC1) was measured. Subsequently, the test tube was autoclaved at 121 ˚C for 20 minutes, and after cooling, the second electrical conductivity (EC2) was measured. Electrolyte leakage was computed as shown in **Equation 1**.

$$\text{Electrolyte leakage} = \frac{\text{EC1}}{\text{EC2}} \text{x} 100 \quad ..... (1)$$

Using an analytical balance, the shoot and root dry weight were determined after oven-drying samples at 70 ˚ C for 48 hours. Analysis of variance (ANOVA) was performed at the $p < 0.05$ significance level. To determine significant differences, mean values were compared using DMRT at the 5% significance level.

RESULTS AND DISCUSSION

Gas Exchange Parameters

Based on data analysis, there was no interaction effect between salinity and AsA concentration. However, gas exchange parameters are significantly ($p<0.05$) affected by salinity (**Table 2**). The treatment of salinity 50 mM caused a decrease in photosynthesis, transpiration, and stomatal conductance by 36.03%, 25.93%, and 33.69%, respectively. In addition, 100 mM of salinity caused a decrease in photosynthesis, transpiration, and stomatal conductance by 48.83%, 37.04%, and 25.11%, respectively. Elevated salinity levels led to a decline in photosynthesis, transpiration, and stomatal conductance. This salinity stress disrupts plants' physiological processes, ultimately hindering their function. Salinity can suppress the rate of gas exchange parameters through various mechanisms.

A reduction in the photosynthesis apparatus may be correlated with a decline in water use efficiency and disturbance of gas exchange (Silva et al., 2023). This was confirmed by Dewi et al. (2023), that transpiration and water use efficiency decreased in high salinity levels. Plants exposed to salt tend to close their stomata, thereby reducing transpiration and carbon dioxide uptake, ultimately decreasing the photosynthesis rate. The subsequent reduction in internal CO_2 concentration diminishes the activity of many enzymes, including Rubisco, limiting carboxylation and consequently reducing the net photosynthetic rate (Hassan et al., 2021). Salinity also causes a decrease in photosystem activity by disrupting the chloroplast lamellar system and damaging chloroplast integrity (Bybordi, 2012). In addition, a decrease in photosynthesis may occur due to chlorophyll degradation caused by the accumulation of Na^+ and Cl^- (Bouzroud et al., 2023). Nawaz et al. (2021) found that salinity hurt gas exchange characteristics in Mung bean plants. However, they also found the function of AsA in H_2O_2 detoxification and as an enzyme co-factor involved in improving plant characteristics.

In the present study, photosynthesis rate, transpiration rate, and stomatal conductance showed an increasing trend at AsA treatments but did not differ from each other (**Table 2**). The highest photosynthesis was found when 400 ppm ascorbic acid was applied, while the highest transpiration and stomatal conductance were when 800 ppm ascorbic acid was added; however, the differences were not significant. Plants treated with AsA exhibited slightly higher average rates of gas exchange parameters compared to AsA control. This shows that ascorbic acid has the potential to improve gas exchange parameters. The improvement in gas exchange characteristics is thought to be caused by AsA enhancing the activity of photosynthetic enzymes like carbonic anhydrase and Rubisco (Siddiqui et al., 2018). Adding exogenous antioxidants can trigger antioxidant activity and its effect on lipid peroxidation and membrane permeability (El-Hawary et al., 2023), thus, it can reduce the adverse effects of salinity. However, a comprehensive investigation is necessary to determine the definitive AsA concentration that optimizes photosynthetic efficiency.

Number of Stomata

Stomata are microscopic pores on leaves that regulate gas exchange and play an essential role in photosynthesis and transpiration. Environmental factors can cause a decrease in the number and function of stomata on plant leaves. In the present study, there was no interaction effect between salinity and AsA concentration. However, salinity levels significantly ($p<0.05$) influence the number of stomata (**Table 2**). The higher the salinity, the number of stomata decreases. The reduction at 50 and 100 mM was 20.74% and 30.86%, respectively, compared with non-saline plants. Salinity-induced osmotic stress is believed to be responsible for decreased stomata density. This stress disrupts cell expansion in young leaves and hinders the formation of stomata (Krismiratsih et al., 2020).

The application of AsA significantly ($p<0.05$) affects the number of stomata. Soybean plants treated with AsA had more stomata (leaf pores) compared to untreated plants. The increase in stomata number in the 400 and 600 ppm AsA treatments was not significantly different from untreated plants. The application of 800 ppm AsA significantly increased the number of stomata, but it was not significantly different from the 600 ppm AsA treatment. This suggests that ascorbic acid might promote stomata formation, possibly by reducing cellular damage and stimulating enzymes that support plant development. According to Xu & Huang (2017), oxidative damage and increased antioxidant enzyme activity can occur with the exogenous application of ascorbic acid.

Table 2. Effect of salinity and exogenous ascorbic acid on the parameters of gas exchange, number of stomata, AsA content, and electrolyte leakage.

Treatments	A	E	*gs*	Number of Stomata	AsA	EL
	(μmol.m^{-2}s^{-1})				AsA.g^{-1} sample	(%)
Salinity levels (S)						
0 mM (control)	3.83 a	0.27 a	6.53 a	18.08 a	7.83 a	6.83 c
50 mM	2.45 ab	0.20 ab	4.33 b	14.33 b	6.88 b	13.95 b
100 mM	1.96 b	0.17 b	4.89 b	12.50 b	6.05 c	21.34 a
Ascorbic Acid concentrations (A)						
0 ppm (control)	2.35 a	0.20 a	4.70 a	13.89 b	7.41 a	14.19 a
400 ppm	3.33 a	0.23 a	5.18 a	14.11 b	6.45 b	13.81 a
600 ppm	2.72 a	0.18 a	5.49 a	15.00 ab	6.38 b	13.56 a
800 ppm	2.59 a	0.24 a	5.61 a	16.89 a	7.43 a	14.60 a
Interaction S x A	ns	ns	ns	ns	6.64	ns

The values in each column with the same letter are not significantly different at $p < 0.05$ by DMRT. A=photosynthesis rate; E=transpiration rate; gs=stomatal conductance; AsA=Ascorbic acid content; EL=Electrolyte Leakage; ns=non-significant.

Electrolyte Leakage

Evaluating electrolyte leakage can help determine membrane damage in plants, and reflect membrane stability. The present study had no interaction effect between salinity and AsA concentration. However, salinity significantly ($p<0.05$) increased electrolyte leakage in plants (**Table 2**). An increase in electrolyte leakage occurred with increasing salinity levels. At a salinity of 50 mM, electrolyte leakage reached 13.95%, while at 100 mM salinity, it reached 21.34%. The lowest electrolyte leakage was observed in the control plants at 6.83%. Plant cell membranes act as selective barriers, controlling the movement of essential nutrients and ions in and out of the cell. These membranes become destabilized when exposed to high salinity (100 mM). This leads to an increase in electrolyte leakage, indicating damage to the membrane. Plant stress may lead to increased electrolyte leakage due to damage to the phospholipid membrane caused by the peroxidation of saturated fatty acids (Khazaei & Estaji, 2020).

Salinity can cause increased electrolyte leakage, but adding AsA can potentially reduce the impact of salinity on plants by suppressing electrolyte leakage. Electrolyte leakage showed a decreasing trend but did not differ from each other. However, the lowest electrolyte leakage value was in the application of AsA 600 ppm (13.56%), while in the control plant, the value was 14.19%. This decrease indicates the potential of ascorbic acid to protect the membrane from damage. While the tested concentration of AsA provided some responses, it wasn't strong enough to fully protect the plant's membranes from damage. According to Alayafi (2020), the application of AsA 0.05 mM can protect membranes and reduce damage caused by oxidative stress, thereby reducing electrolyte leakage.

Ascorbic Acid Content

Based on the analysis results, salinity levels, AsA concentrations, and their interactions significantly ($p<0.05$) affect AsA content. Endogenous ascorbic acid was significantly affected by salinity levels. At high salinity levels, endogenous ascorbic acid showed a reduction. The decrease in AsA content at 50 mM of salinity was 12.13%, while at 100 mM of salinity, it decreased by 22.73% (**Table 2**). This is in line with Billah et al. (2017), that salinity can reduce endogenous AsA content. The decrease in AsA content may be due to inhibition of AsA synthesis. Wang et al. (2024) stated that stress can reduce AsA synthesis caused by downregulation of expression genes that are important for ascorbic acid synthesis.

The application of exogenous ascorbic acid significantly affected plant AsA content. At 400 ppm and 600 ppm concentrations, AsA content decreased by 12.95% and 13.90%, respectively, compared to the control. Meanwhile, at 800 ppm, it slightly increased by 0.27% but was not significantly different from control plants. Ascorbic acid has a multifunctional role in increasing plant tolerance to salinity. In addition to strengthening cell membranes, ascorbic acid also stimulates the production of compounds that act as antioxidants and osmoprotectants (Kanwal et al., 2024). Thus, ascorbic acid is needed to protect cellular components from oxidative damage and help plants maintain water balance and metabolic functions under salinity conditions.

Shoot and Root Dry Weight

Plant physiological processes can influence biomass accumulation. This is apparent in the dry weight of the shoots and roots. The present study revealed no interactive effect of salinity levels and AsA concentration on shoot dry weight. However, an interaction effect was observed between salinity levels and AsA concentration on root dry weight.

The dry weight of shoots and roots was significantly ($p<0.05$) influenced by a single factor of salinity, the weight decreased with increasing salinity levels (**Table 3**). Shoot dry weight decreased by 23.15% and 27.96% at salinity 50 mM and 100 mM, respectively, compared to nonsalinity; meanwhile, root dry weight decreased by 26.38% and 34.25%. The lowest shoot and root weights were in the 100 mM salinity, namely 13.32 g and 1.67 g, respectively. This shows that plant growth and development under salinity stress are disrupted. Several factors are likely responsible for this phenomenon, including reduced efficiency of photosynthesis and damage to plant cells. According to Hasanuzzaman et al. (2023) salinity disrupts carbohydrate translocation and the production of photo-assimilation (sugar produced through photosynthesis). Plants with limited photo-assimilation production capacity will have difficulty accumulating biomass Huanhe et al. (2024). Therefore, the dry weight of shoots and roots decreases.

Plants grown under salinity stress tend to have lower weights, with the rate of decline determined by the plant's tolerance to salinity (Nur et al., 2024).

Table 3. The effect of salinity and exogenous ascorbic acid on shoot and root dry weight

Treatments	Shoot	Root
	(gram)	
Salinity levels (S)		
0 mM (control)	18.49 a	2.54 a
50 mM	14.21 b	1.87 b
100 mM	13.32 c	1.67 b
Ascorbic Acid concentrations (A)		
0 ppm (control)	15.11 a	1.65 a
400 ppm	15.58 a	2.16 b
600 ppm	15.53 a	2.11 b
800 ppm	15.13 a	2.19 b
Interaction S x A	ns	3.011

The values in each column with the same letter are not significantly different at $p < 0.05$ by DMRT. ns=non-significant.

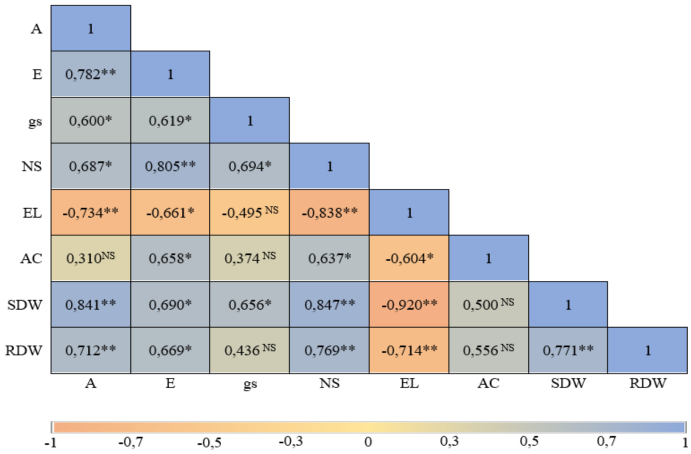
The application of ascorbic acid increases shoot and root dry weight. The increase in roots was significantly different from control plants, while the shoots were not significantly different from each other. The highest value of shoot dry weight was in the AsA treatment of 400 ppm (15.58 g), but showed no significant difference compared to untreated plants. While the highest value of root dry weight was in the AsA treatment of 800 ppm (2.19 g), it did not differ significantly from the 400 ppm of AsA. It was observed that giving exogenous AsA could help plants increase growth and development, thereby increasing dry weight. AsA is thought to overcome salinity stress by increasing several enzyme activities and reducing damage to physiological functions. Generally, ascorbic acid applied foliarly increases plant growth by increasing water status and nutrient uptake (Hasanuzzaman et al., 2023).

Pearson Correlation Analysis

The results of the correlation analysis are shown in **Figure 1**. Based on the analysis results, root dry weight has a significant positive correlation with the parameters of photosynthesis rate, transpiration rate, number of stomata, and shoot dry weight, and has a significant negative correlation with electrolyte leakage. This shows that an increase in root dry weight was accompanied by an increase in photosynthesis rate, transpiration rate, number of stomata, and shoot dry weight. In addition, a decrease in root dry weight accompanied an increase in electrolyte leakage. Root dry weight has a positive but insignificant correlation with stomatal conductance and ascorbic acid content.

There is a strong positive correlation between root dry weight and photosynthesis rate (r=0.712), number of stomata (r=0.769), and shoot dry weight (r=0.771). Additionally, it has a strong negative correlation with electrolyte leakage (r=0.714). Stomata serve as portals for gas exchange, which is essential for photosynthesis. High photosynthetic rates lead to increased plant biomass production, part of which is allocated to root growth. Furthermore, electrolyte leakage indicates cell damage, suggesting healthier roots are more resistant to stress.

e-ISSN 2655-853X
https://doi.org/10.37637/ab.v7i3.1948



	A	E	gs	NS	EL	AC	SDW	RDW
A	1							
E	0,782**	1						
gs	0,600*	0,619*	1					
NS	0,687*	0,805**	0,694*	1				
EL	-0,734**	-0,661*	-0,495^{NS}	-0,838**	1			
AC	0,310^{NS}	0,658*	0,374^{NS}	0,637*	-0,604*	1		
SDW	0,841**	0,690*	0,656*	0,847**	-0,920**	0,500^{NS}	1	
RDW	0,712**	0,669*	0,436^{NS}	0,769**	-0,714**	0,556^{NS}	0,771**	1

Figure 1. Pearson correlation coefficients analysis

Where A=photosynthesis rate, E=transpiration rate, gs=stomatal conductance, NS=number of stomata, EL= Electrolyte Leakage, AC=Ascorbic acid content, SDW=shoots dry weight, RDW=roots dry weight. ** = Correlation is significant at the 1% level, * = Correlation is significant at the 5% level, ^{NS} = non-significant at the 5% level

Electrolyte leakage has a significant negative correlation with photosynthesis rate, transpiration rate, number of stomata, AsA content, shoot, and root dry weight. In addition, electrolyte leakage has a negative but insignificant correlation with stomatal conductance. This shows that with the increase in electrolyte leakage, these parameters will show a decrease. Electrolyte leakage has a strong negative correlation with photosynthesis rate (r= 0.734), number of stomata (r= 0.838), and root dry weight (r= 0.714), and has a very strong negative relationship with shoot dry weight (r= 0.920). This indicates that damage to the cell membrane can disrupt the photosynthesis process. Additionally, cell damage can reduce shoot growth and interfere with stomata formation.

CONCLUSION

Salinity reduced photosynthesis rate, transpiration rate, stomatal conductance, leaf ascorbic acid content, number of stomata, and plant biomass. Additionally, electrolyte leakage increased under saline conditions. Exogenous ascorbic acid significantly increased the number of stomata and root dry weight. Still, it did not significantly increase the photosynthesis rate, transpiration rate, stomatal conductance, and shoot dry weight, nor did it reduce membrane damage. However, 400 ppm ascorbic acid could improve physiological and biomass accumulation. While AsA shows potential in alleviating salinity stress, further research is necessary to determine the optimal concentration, application method, and treatment frequency.

ACKNOWLEDGEMENTS

The author would like to thank the Agricultural Extension and Human Resources Development Agency, the Ministry of Agriculture Republic of Indonesia, for their support of this study.

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