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Enhancing Salinity Resilience in *Vigna radiata* L. through Brassinolide and α -Tocopherol-Mediated Oxidative Stress Regulation.

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Keywords	Abstract
Salinity, MDA, H ₂ O ₂ , NaCl.	Salinity stress disrupts cellular redox homeostasis, leading to an imbalance in the production and scavenging of reactive oxygen species (ROS). This imbalance primarily arises from disturbances in the electron transport chains during photoinhibition and from reduced cellular water potential. Under saline conditions, ROS levels increase rapidly. Excessive accumulation of ROS can become cytotoxic, causing damage to cellular components and potentially leading to cell death if not effectively regulated. In the present investigation, a pot culture experiment was carried out to examine the effects of sodium chloride (NaCl) stress and the potential ameliorative role of the plant growth regulators brassinolide and α -tocopherol in <i>Vigna radiata</i> L. The growth regulators were administered through foliar spraying, with brassinolide applied at a concentration of 4 mg L ⁻¹ and α -tocopherol at 200 mg L ⁻¹ ,



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whereas salinity stress was imposed by soil drenching with 80 mM NaCl solution. The experimental plants were arranged into six treatment groups: control, NaCl alone, NaCl combined with brassinolide, NaCl combined with α -tocopherol, brassinolide alone, and α -tocopherol alone. Plant samples were collected randomly on the 25th, 35th, and 45th days after sowing for the assessment of various parameters. The results indicated that foliar supplementation with α -tocopherol and brassinolide significantly alleviated the adverse effects of salinity stress in *Vigna radiata*. This mitigation was evidenced by a reduction in oxidative stress indicators, particularly malondialdehyde (MDA) and hydrogen peroxide (H_2O_2), suggesting an improved oxidative balance and enhanced stress tolerance in treated plants.

1. Introduction

Global agriculture is currently confronted with significant challenges, particularly the necessity to increase food production by nearly 70% to meet the demands of a rapidly expanding global population. However, crop productivity is not rising at a rate sufficient to match this escalating food requirement. One of the primary factors responsible for reduced agricultural output is the prevalence of various abiotic stresses that adversely affect plant growth and development. Minimizing crop losses caused by unfavorable environmental conditions has therefore become a critical priority for ensuring future food security (Shanker and Venkateswarlu, 2011). Among these constraints, major abiotic stress factors including salinity, drought, extreme temperatures such as heat and cold severely impair plant survival, limit biomass accumulation, and significantly reduce the yield potential of major staple crops, in some cases causing productivity losses of up to 70%. (Mantri *et al.*, 2011). Salinity stress elicits a wide range of responses in plants, encompassing morphological, physiological, biochemical, and molecular alterations. Elevated salt concentrations disrupt cellular ionic homeostasis, leading to ionic toxicity and osmotic stress. (Ambede *et al.*, 2012) In addition, salinity stress promotes the excessive generation of reactive oxygen species (ROS) within plant cells. The overaccumulation of ROS induces oxidative stress, which can severely damage essential cellular components, including membrane lipids, proteins, and nucleic acids. (Abreu *et al.*, 2013). Elevated concentrations of NaCl in plant roots have been associated with enhanced oxidative stress, as evidenced by increased levels of stress indicators such as electrolyte leakage (EL), malondialdehyde (MDA), and hydrogen peroxide (H_2O_2). Concurrently,



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the redox status of important non-enzymatic antioxidants, including reduced glutathione (GSH) and ascorbate (ASC), is significantly altered in root cells (Ghosh *et al.*, 2015). Severe salinization has been widely reported to induce oxidative stress across multiple plant tissues and cellular compartments. Excess electrochemical energy may be degenerated by the Mehler reaction, which is a major cause of ROS and H₂O₂ production. Membrane injury is revealed by prominent EL and MDA levels. (Sharma *et al.*, 2012). similar reports were found in the experimental investigation, the salinity stress enhanced the concentration of oxidative markers like MDA and hydrogen peroxide in case of leaf, stem and root.

2. Experimental design

The experimental study was conducted in the Botanical Garden of the Department of Botany, Annamalai University, Tamil Nadu, India. The geographical coordinates of the experimental site are 11°23'23.1" N latitude and 79°43'05.3" E longitude. Healthy and uniform seeds were initially surface-sterilized using a 0.2% (w/v) mercuric chloride (HgCl₂) solution for two minutes, followed by repeated rinsing with double-distilled water (ddH₂O) to eliminate any residual sterilant. A total of 90 pots were prepared and arranged into six experimental groups. Each pot was filled with a homogenized soil mixture comprising red soil, sand, and well-decomposed farmyard manure in a ratio of 1:1:1. The plants were subjected to the following treatments: control (untreated), 80 mM NaCl, 80 mM NaCl + 4 mg L⁻¹ brassinolide (BL), 80 mM NaCl + 200 mg L⁻¹ α -tocopherol, 4 mg L⁻¹ brassinolide alone, and 200 mg L⁻¹ α -tocopherol alone. To ensure the maintenance of the desired salinity level, soil electrical conductivity was periodically monitored using an electrical conductivity (EC) meter. Plants were harvested at three different growth stages 25, 35, and 45 days after sowing (DAS) for subsequent experimental analyses. These sampling intervals were selected to evaluate the influence of the various treatments on plant growth and associated biochemical attributes.

3. METHODOLOGY

3.1 Malondialdehyde (MDA) (Heath and Packer, 1968)

The end-product of peroxidation of polyunsaturated fatty acids, i.e., malondialdehyde (MDA) was estimated following the thiobarbituric acid (TBARS) reaction as described by (Heath and Packer, 1968).



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0.5g of fresh plant tissue (FW) was homogenized in 5 ml of 0.1% (w: v) TCA. The homogenate was centrifuged at 10,000 xg for 5 minutes at 4°C temperature. From this 1 ml of the supernatant was taken, and 4ml of 20% TCA containing 0.5 % (w: v) TBA was added as a reagent. The mixture was incubated at 95°C in a shaking water bath for 30 minutes and then quickly cooled on an ice bath. The contents were again centrifuged at 10,000xg for 15 min. and then absorbance was read at 532 nm in a spectrophotometer. The concentration of MDA was calculated using an extinction coefficient of 155mM⁻¹ cm⁻¹ and expressed as nmol g⁻¹ FW.

$$\text{MDA level (nmole)} = \Delta (A_{532\text{nm}} - A_{600\text{nm}}) / 1.56 \times 10^5$$

3.2 Hydrogen peroxide (Velikova *et al.*, 2000)

Hydrogen peroxide level in the samples was determined following the method of Velikova *et al.*, (2000).

Fresh leaf tissue (0.5gm) was ground in a pre-chilled mortar with 5 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 rpm for 15 min. To 0.5 ml of the supernatant, 0.5 ml of 10mM potassium phosphate buffer (pH 7.0) and 1 ml of 1M KI were added. After vortexing, the mixture its absorbance was read at 390 nm. The hydrogen peroxide content was calculated based on a standard curve made by measuring known hydrogen peroxide concentrations ranging from 0 to 1,000 μ M in the same assay. The concentration of H₂O₂ was expressed as μ mol. g⁻¹ FW.

4.RESULTS

4.1 Malondialdehyde (MDA) content- (Leaf)

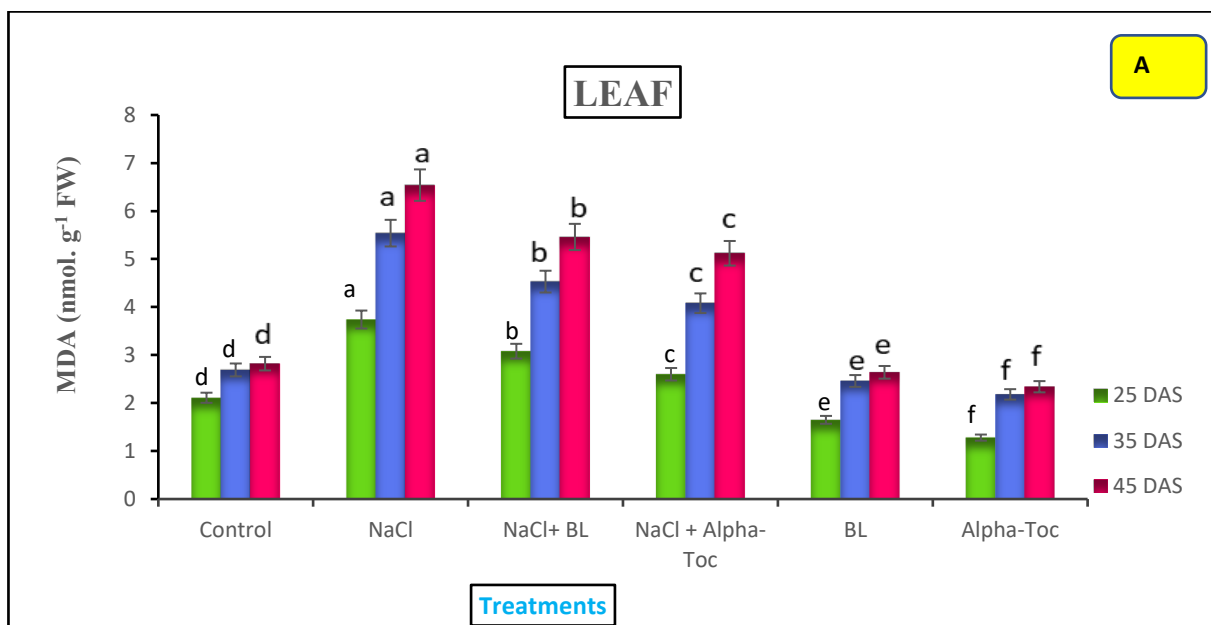
The MDA content in the leaves of NaCl-stressed plants was higher than in the control plants on all sampling days, showing increases of 177.25, 205.94, and 231.91 Percent over control at 25, 35, and 45 DAS, respectively. However, the foliar application of Brassinolide (BL) and Alpha-tocopherol (Alpha-toc) under salt stress conditions reduced MDA levels compared to NaCl-only treated plants, although these levels remained higher than the control, with values of 193.61% and 181.50% above



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the control at 45 DAS, respectively. In unstressed plants, BL and Alpha-toc treatments also decreased MDA content relative to the control, with reductions of 93.61 and 84.02 percent compared to control at 45 DAS (figure 1 A).

Figure 1 A: Effect of exogenous application of Brassinolide (BL) and Alpha-tocopherol (Alpha-toc) on Malondialdehyde (MDA) content of *Vigna radiata* L. under NaCl stress. (Values are expressed in nmol. G⁻¹ FW)



The values are the mean $\pm$ SE of the Seven replicates. The lowercase letters above the bars not sharing a common superscript (a, b, c, d, e, f) between the treatments differ significantly according to one-way ANOVA (Duncan's Test- $p \leq 0.05$)

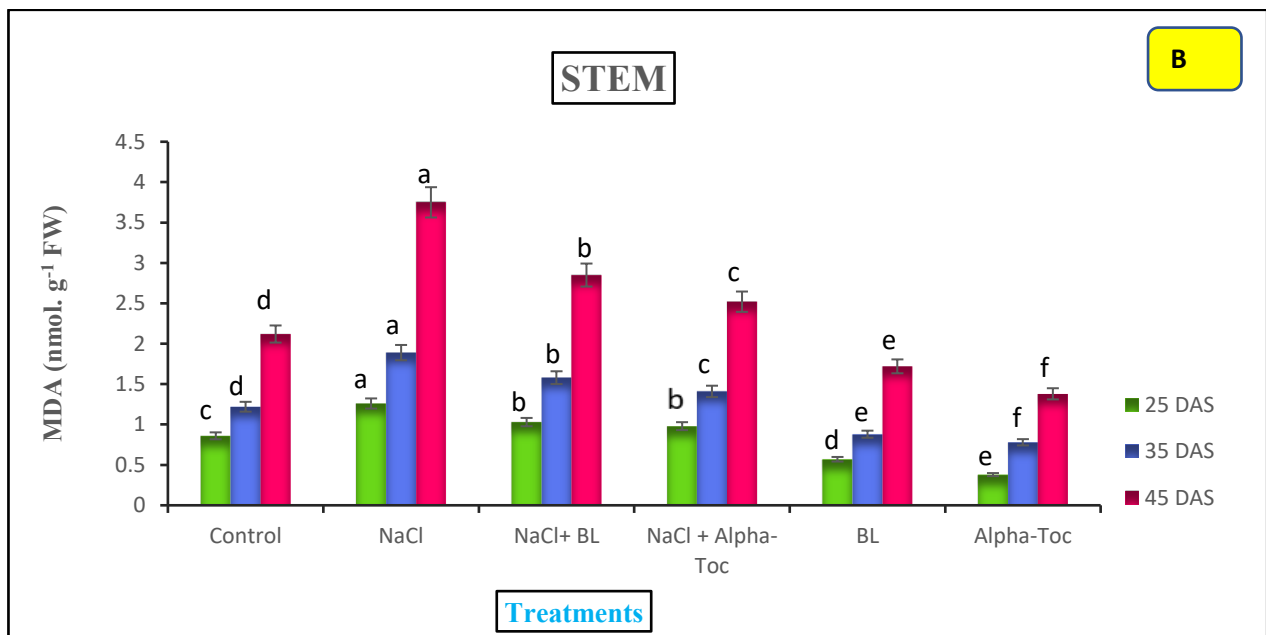
Stem

In salt-stressed plants, MDA activity in the stem increased compared to the control and other treatments, recorded 176.88 percent compared to control at 45 DAS. When salt-stressed plants were treated with Brassinolide (BL) and Alpha-tocopherol (Alpha-toc), the MDA content of stem was reduced to 134.43 and 118.86 percent over the control at 45 DAS, though these values were still higher than in the control plants. Additionally, the application of BL and Alpha-toc to unstressed plants also

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resulted in reduced stem MDA content, measuring 81.13 and 65.09 percent over control at 45 DAS (Figure -2 B).

Figure 2 B: Effect of exogenous application of Brassinolide (BL) and Alpha-tocopherol (Alpha-toc) on Malondialdehyde (MDA) content of *Vigna radiata* L. under NaCl stress. (Values are expressed in nmol. G⁻¹ FW)



The values are the mean $\pm$ SE of the Seven replicates. The lowercase letters above the bars not sharing a common superscript (a, b, c, d, e, f) between the treatments differ significantly according to one-way ANOVA (Duncan's Test- $p \leq 0.05$)

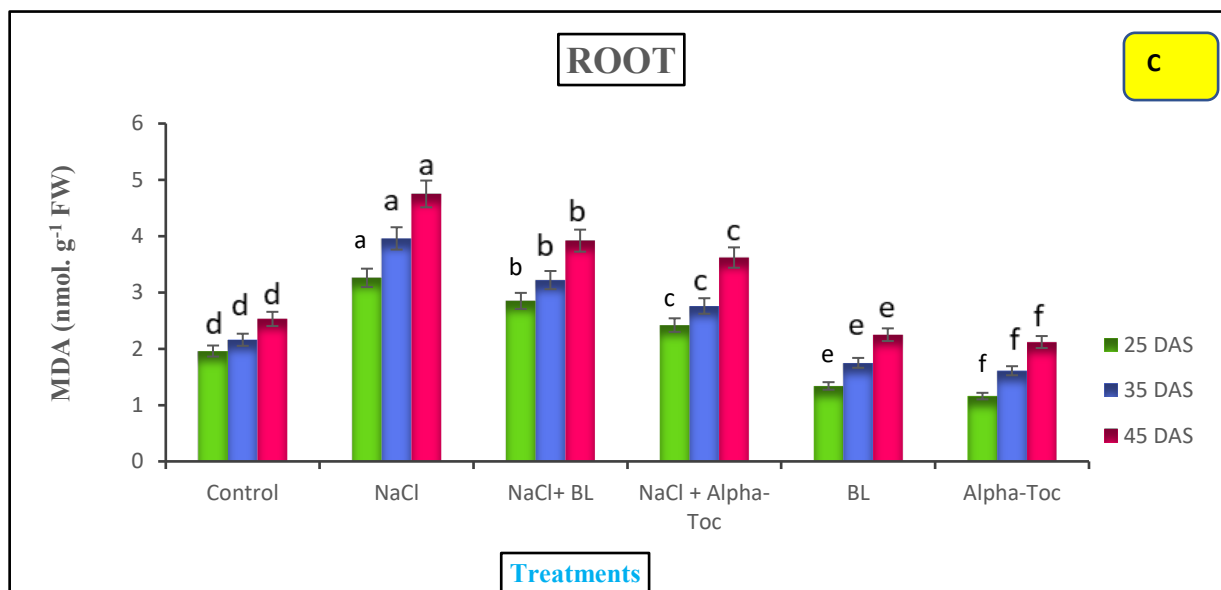
Root

Salt stress increased MDA levels in the roots of green gram plants compared to the control and other treatments at all growth stages, with values of 166.32, 183.34, and 187.74 percent over the control at 25, 35, and 45 DAS, respectively. However, treating salt-stressed plants with Brassinolide (BL) and Alpha-tocopherol (Alpha-toc) significantly reduced root MDA levels, measuring 154.94% and 143.08% over the control at 45 DAS, although these levels remained higher than the control. Additionally, in unstressed plants, BL and Alpha-toc treatments also reduced root MDA levels

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compared to the control, with values of 88.93 and 83.79 percent over the control at 45 DAS (Figure-3 C).

Figure 3 C: Effect of exogenous application of Brassinolide (BL) and Alpha-tocopherol (Alpha-toc) on Malondialdehyde (MDA) content of *Vigna radiata* L. under NaCl stress. (Values are expressed in nmol. G⁻¹ FW)



The values are the mean $\pm$ SE of the Seven replicates. The lowercase letters above the bars not sharing a common superscript (a, b, c, d, e, f) between the treatments differ significantly according to one-way ANOVA (Duncan's Test- $p \leq 0.05$)

4.2 Hydrogen peroxide (H₂O₂)

Leaf.

Salt-stressed plants showed an increase in H₂O₂ content in the leaves compared to the control and other treatments on all sampling days, reaching 201.04 percent at 45 DAS. When salt-stressed plants were treated with Brassinolide (BL) and Alpha-tocopherol, the H₂O₂ content in the leaves decreased to 174.12 and 160.13 percent over the control at 45 DAS, although these levels were still higher than the

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control. Additionally, BL and Alpha-toc treatments in unstressed plants also reduced H_2O_2 content compared to the control, with values of 82.51 and 69.23 percent over the control at 45 DAS (Table 1).

Stem

In salt-stressed plants, H_2O_2 content in the stem increased compared to the control on all sampling days, reaching 180.59, 173.50 and 172.07 percent over the control at 25, 35, and 45 DAS, respectively. However, foliar application with Brassinolide (BL) and Alpha-tocopherol (Alpha-toc) under salt stress conditions reduced stem H_2O_2 levels compared to untreated salt-stressed plants, although the levels remained higher than the control, at 150.18 and 138.49 percent over the control at 45 DAS. Additionally, BL and Alpha-toc treatments in non-stressed plants also lowered stem H_2O_2 content, recorded 86.03 and 69.43 percent over the control at 45 DAS (Table 1).

Root

NaCl stress increased hydrogen peroxide content in the roots of green gram plants compared to the control, with values of 171.30, 174.80, 188.58 percent over the control on the respective sampling days of 25, 35, and 45 DAS. However, salt-stressed plants treated with exogenous Brassinolide (BL) and Alpha-tocopherol (Alpha-toc) exhibited a significant decrease in root H_2O_2 content compared to untreated salt-stressed plants, although these levels remained higher than the control, measuring 172.37 and 159.75 percent over the control at 45 DAS, respectively. Further BL and Alpha-toc treatments in non-stressed plants also lowered root H_2O_2 content, recorded 96.69 and 88.89 percent compared to control. (Table 1).



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DA	Treatments					
	Control	NaCl	NaCl + BL	NaCl + Alpha-Toc	BL	Alpha-Toc
LEAF						
25	1.88 ± 0.039d	3.55 ± 0.049a [188.82]	2.62 ± 0.049b [140.86]	2.32 ± 0.073c [123.40]	1.56 ± 0.061e [82.97]	1.24 ± 0.076f [65.95]
35	2.52 ± 0.079d	4.94 ± 0.018a [196.03]	3.68 ± 0.094b [146.03]	3.36 ± 0.084c [133.34]	1.98 ± 0.099e [78.57]	1.68 ± 0.065f [66.67]
45	2.86 ± 0.047d	5.71 ± 0.056a [201.04]	4.98 ± 0.047b [174.12]	4.58 ± 0.065c [160.13]	2.34 ± 0.069e [82.51]	1.98 ± 0.066f [69.23]
STEM						
25	1.34 ± 0.046c	2.42 ± 0.072a [180.59]	1.72 ± 0.066b [128.35]	1.676 ± 0.061b [125.37]	0.92 ± 0.099d [68.65]	0.72 ± 0.047e [53.73]
35	2.32 ± 0.063d	4.06 ± 0.085a [173.50]	3.36 ± 0.088b [143.58]	3.22 ± 0.04c [137.60]	1.95 ± 0.079e [84.61]	1.62 ± 0.042f [69.82]
45	2.65 ± 0.085d	4.56 ± 0.054a [172.07]	3.98 ± 0.081b [150.18]	3.63 ± 0.089c [138.49]	2.28 ± 0.079e [86.03]	1.86 ± 0.047f [70.18]
ROOT						
25	2.23 ± 0.066d	3.82 ± 0.037a [171.30]	3.34 ± 0.046b [149.77]	2.98 ± 0.081c [133.63]	1.85 ± 0.035e [82.95]	1.64 ± 0.034f [73.54]
35	2.62 ± 0.072d	4.58 ± 0.088a [174.80]	4.02 ± 0.047b [153.43]	3.68 ± 0.034c [140.45]	2.32 ± 0.081e [88.54]	1.84 ± 0.034f [70.29]
45	3.33 ± 0.075d	6.28 ± 0.04a [188.50]	5.74 ± 0.064b [172.37]	5.32 ± 0.102c [159.75]	3.22 ± 0.042d [96.69]	2.96 ± 0.047e [88.89]

5. DISCUSSION

The MDA content in *Vigna radiata* L. plants subjected to NaCl stress increased with the duration of the stress, compared to plants that were not stressed. Our observations of Increased MDA levels align with those reported by previous researchers (Aljuaid, and Ashour 2022) and Maize (Çirka *et al.*, 2022). Further Malondialdehyde (MDA) content acts as a significant marker for lipid peroxidation in plants under salt stress, indicating oxidative damage (Ogunsiji, *et al.*, 2023). Additionally, elevated levels of H₂O₂ and MDA in plants under salinity stress are believed to enhance mechanical strength and decrease cell wall extensibility, thereby restricting plant growth and expansion. (Rahman *et al.*, 2016). The application of Brassinolide and Alpha -tocopherol significantly reduced MDA levels in all tissues as in root, stem, and leaves of green gram plants subjected to 80mM NaCl treatment alone. This reduction was greater than that observed in control plants. Additionally, a significant decrease in MDA levels was also noted in non-saline plants treated with exogenous BL. Our results align with the results of (Maia *et al.*, 2022) who reported that foliar application of epibrassinolide (EBL) mitigates the negative impacts of salinity on tomato plants by lowering MDA content and enhancing membrane integrity, under salt stress conditions. Moreover, BL treatment enhances antioxidant capacity to counteract the excessive buildup of ROS (Hafeez *et al.*, 2021). Further, more in accordance with the results we found, earlier research indicates that



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exogenously applied Alpha-tocopherol enhances membrane tolerance and reduces MDA content under abiotic stress. Viz (Akram *et al.*, 2023) *Solanum melongena* L. and (Sadiq *et al.*, 2018) *Vigna radiata* L. the improvement in membrane integrity is attributed to the increase in antioxidant levels resulting from the exogenous application of Alpha-tocopherol. However, various studies indicate that while Alpha-tocopherol alone may slightly elevate MDA content and is not essential for plant regulation. (Akram *et al.*, 2023)

In our study, application of NaCl to green gram plants resulted in a significant rise in H₂O₂ content in the roots and shoots (stems and leaves) in comparison to the control plants, as the stress period extended. Our results were supported by the results of (Farhangi-Abriz and Torabian, 2017), in common bean, Kamanga *et al.*, (2020) in tomato varieties, reported that the NaCl stress increases the H₂O₂ content in plants. Further The foliar application of Brassinolide (BL) significantly reduced H₂O₂ content in all parts of salt-treated plants, with the decrease becoming more pronounced over time and with extended salt exposure, compared to plants exposed to salt stress alone. Nevertheless, the H₂O₂ levels remained higher than those observed in the control group. our results are very aligning with the previous results of (Silva *et al.*, 2023), who reported that brassinolide treatment lower H₂O₂ levels by boosting the antioxidant system, which includes increasing the activities of antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APOX). Furthermore, the subsequent application of Alpha-tocopherol to NaCl-treated plants resulted in decreased H₂O₂ levels in all tissues compared to plants subjected to NaCl stress alone. However, the reduction was still greater than that observed in the control group. results align with the results of kao and Ching-huei., (2014) who reported that hydrogen peroxide can mitigate the H₂O₂ levels under salt stress. Further other authors also reported a decrease level of H₂O₂ on the application of Alpha-tocopherol (Semida *et al.*, 2016) in *Allium cepa* (Hemida *et al.*, 2017).

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AUTHOR(S) CONTRIBUTION

The writers affirm that they have no connections to, or engagement with, any group or body That provides financial or non-financial assistance for the topics or resources covered in this Manuscript.

CONFLICTS OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, And/or publication of this article.

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