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Efficiency of seed priming and co-treatment strategies in salt effect mitigation using *Nicotiana glauca* leaf extract on tomato seedlings (*Solanum lycopersicum* L.)

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Abstract. The Al-Baha region suffers from soil salinity, negatively impacting agriculture. Current study examined aqueous extracts from *Nicotiana glauca* as plant-based biostimulants to alleviate salinity's effects and reduce chemical fertilizer use. An aqueous extract of *N. glauca*, applied in ascending doses of 20%, 50%, and 100%, was being used. This biostimulant was applied using two methods: seed priming and co-treatment on the seeds of *Solanum lycopersicum* L. Results indicated that all tested doses, whether applied through seed priming or co-treatment, enhanced the final germination percentage (FGP) and reduced the mean germination time (MGT) under saline conditions. With priming, the 20% dose was most effective in reducing salt treatment effects on FGP, improving it by 5% compared to untreated salt-stressed seeds. In co-treatment, the 100% dose showed a 4.5% reduction in FGP decline referring to untreated and stressed seeds. For priming treatments, the 20% dose reduced MGT by 22%, while the 50% dose in co-treatment reduced it by 28% compared to untreated and stressed seeds. The priming strategy used in this study did not result in significant enhancements in growth parameters, particularly in the fresh weight (FW) and dry weight (DW) of the epicotyls. As priming, the 20% dose had the most significant mitigation effect on epicotyl FW, DW and chlorophyll (Chl) content by 15%, 10% and 30% referring to untreated and stressed seedlings, respectively. However, all tested biostimulant doses used as co-treatment proved effective in mitigating the negative effects of salt on epicotyl and root FW, DW, and Chl content. Seedlings treated with various strategies and doses showed a smaller increase in malondialdehyde (MDA) and proline (Pro) levels under salinity stress. As stress indicators, both Pro and MDA levels were significantly reduced when a co-treatment strategy was applied. Furthermore, the results indicated that the effectiveness of the *N. glauca* aqueous extract in alleviating salt stress could be attributed to its content of several phenolics, flavonoids, and tannins, which possess antioxidant properties that enhance the plant's tolerance against salt-induced oxidative stress.

Key words: antioxidant activity, biostimulant, flavonoids, germination, polyphenolics, tannins, tree tobacco, salinity.

INTRODUCTION

Climate change has affected enormous agriculture areas worldwide through soil salinization (Eswar et al., 2021). Farmers have implemented a variety of strategies to address agricultural production challenges in saline soils, particularly through the use of chemical fertilizers (Sharma & Chetani 2017). With the tremendous use of the chemical fertilizer, the soil physicochemical parameters have been seriously affected reducing soil fertility. To address this challenge, a range of natural substances has been employed as biostimulants (Pascual et al., 2021; Kumari et al., 2022; Chanthini et al., 2024; Sarov et al., 2024).

The use of plant-based biostimulants to mitigate salinity can be regarded as an environment friendly and sustainable way of fighting abiotic stress, as it contains no synthetic chemicals. Chernikova et al. (2021) reported that biostimulant effectiveness depend strongly on type of soils. Depending upon the plant species and plant part used to prepare biostimulants, it may contain various amounts of bioactive compounds such as flavonoids, phenolics, tannins, glycosides, phytohormones, mineral elements and photosynthetic pigments. Several bioactive components had a potential antioxidant capacity; therefore, it's deduced that they might also be effective in plants against salinity as it disrupts redox balance in plants. This has been demonstrated in previous studies that plant-based biostimulants contribute to a better growth, development, yield, disease and stress-resistance in plants given the presence of aforementioned compounds (Howladar, 2014; Drobek et al., 2019; Desoky et al., 2020; Zulfiqar et al., 2020). Polyphenols, flavonoids, and tannins are the largest groups of specialized metabolites found in plants, and they are widely recognized for their role in salt stress protection (Kiani et al., 2021). This study suggests that applying external *N. glauca* extract containing polyphenols and flavonoids may help alleviate the effects of salt stress on tomato plant. Nevertheless, sources of biostimulants, methods of application, doses and their implications against salinity should be investigated.

Tomato production is vital in Saudi Arabia, playing a crucial role in ensuring food security, supporting the agricultural sector, and contributing to the economy. However, tomato crops face significant challenges due to soil salinity (Alshami et al., 2023). On the other hand, *Nicotiana glauca*, commonly known as tree tobacco, is an invasive species in Saudi Arabia that presents both ecological and agricultural challenges (Alharthi et al., 2021). Despite its invasive nature, tree tobacco is rich in bioactive compounds, including polyphenolics, flavonoids, and tannins, which contribute to its antioxidant properties (Alghamdi et al., 2021). Recent studies have highlighted its potential as a source of natural antioxidants, showing that extracts from *N. glauca* possess significant free radical scavenging activity. Research by Khedr et al., 2024 indicated that the leaves of *N. glauca* have a high phenolic content, suggesting its potential for use in pharmaceutical and agricultural applications. Jmii et al. (2022) demonstrated that *N. glauca* is abundant in allelochemicals, which may be utilized to manage the invasive weed *Cynodon dactylon* and enhance tomato growth.

The objective of current study was to investigate the biochemical characteristics of the tree tobacco. Specifically, the analysis of the contents of polyphenols, flavonoids, and tannins in the aqueous extract of *N. glauca*, as well as to examine its effects on mitigating salt stress in tomato seedlings using two strategies of biostimulant application with ascending doses.

MATERIALS AND METHODS

Plant materials used as bio-stimulants

Collection of plant material Fresh *Nicotiana glauca* leaves (fronds) were picked at random from the Al-Baha area in the southeastern of Saudi Arabia. Fifteen middle leaves were collected in the morning (7:00–10:00 am) from thirty-five different shrub samples (1.7–2 m). The fresh collected leaves were cleaned, shade dried, then pulverized with a blender and kept in airtight bottles.

Aqueous extract preparation (used as biostimulant). Fifty gram of leaf powder was soaked in 100 mL of distilled water. The suspension was shaken for 24 hours at room temperature (25 °C). Then, it was centrifuged at 3,000 X g for 30 min at 4 °C. Then, the extract was filtered using double layers of Whatman filter paper No. 1. The leaf extract was kept in a refrigerator at 4 °C. The aqueous extract was diluted with distilled water to give 20, 50 and 100% solutions.

Determination of total phenolic compounds. The total phenolic content was determined using the Folin–Ciocalteu method as described by (Attard, 2013). Briefly, the procedure consisted of mixing 10 µL of sample/standard with 100 µL of Folin-Ciocalteu reagent (Diluted 1:10) in a 96-well microplate. Then, 80 µL of 1M Na_2CO_3 was added and incubated at room temperature (25 °C) for 20 min in the dark. At the end of incubation time, the resulting blue complex color was measured at 630 nm. A gallic acid stock solution of 2 mg mL^{-1} in methanol was used as standard, and the following dilutions were prepared: 1,000, 500, 250, 125, 62.5, and 31.25 µg mL^{-1}. Data are represented as means ± SD.

Determination of total flavonoids. The total flavonoid content was determined using the aluminum chloride method as described by (Kiranmai et al., 2011), with minor modifications to be carried out in microplates. Briefly, 15 µL of sample/standard was placed in a 96-well microplate, then, 175 µL of methanol was added followed by 30 µL of 1.25% $AlCl_3$. Finally, 30 µL of 0.125 M $C_2H_3NaO_2$ was added and incubated for 5 min. At the end of incubation, the resulting yellow color was measured at 420 nm. A stock solution of standard Rutin was prepared at 2,000 µg mL^{-1} in methanol, from which the following dilutions were prepared: 1,000, 500, 250, 125, and 62.5 µg mL^{-1}. Data are represented as means ± SD.

Determination of total tannins. The total tannin content was determined using the method of Ojha et al., 2018 with minor modifications to be carried out in microplates. Briefly, 20 µL of sample/standard was placed in a 96-well microplate, then, 20 µL of 1% $FeCl_3$ was added followed by 20 µL of 1% $K_4[Fe(CN)_6]$. Finally, the volume was completed to 200 µL with distilled water and the mixture was incubated for 5 min. At the end of incubation, the resulting yellow color was measured at 720 nm. A stock solution of standard tannic acid was prepared at 2,000 µg mL^{-1} in distilled water, from which the following dilutions were prepared: 1.5625, 3.125, 6.25, 12.5, and 25 µg mL^{-1}. Data are represented as means ± SD.

Determination of antioxidant activity of *N. glauca* extract by DPPH assay. *N. glauca* leaf powder was dissolved in distilled water with final concentrations of 15.625, 31.25, 62.5, 125 and 250 µg mL^{-1} A stock solution of 1,000 µg mL^{-1} concentration of Trolox was prepared in methanol from which 5 concentrations were prepared

including 3.125, 6.25, 12.5, 18.75 and 25 μg mL^{-1}. DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical assay was carried out according to the method of Boly et al., 2016 adopting the modifications of Elkholy et al., 2023. Briefly, 100 μL of freshly prepared DPPH reagent (0.1% in methanol) were added to 100 μL of the sample in 96 wells plate (n = 3), the reaction was incubated at room temperature for 30 min in the dark. At the end of incubation, the resulting reduction in DPPH color intensity was measured at 540 nm. Data are represented as means ± SD according to the following equation: percentage inhibition = ((Average absorbance of blank-average absorbance of the test)/ (Average absorbance of blank)) *100). Data were normalized using Microsoft Excel and the IC50 value was calculated using Graph pad Prism 9., the concentrations were converted into logarithmic values, and a non-linear inhibitor regression equation: (log (inhibitor) vs. normalized response - variable slope equation) was selected.

Qualitative HPLC analysis of polyphenolics and flavonoids. A Waters 2690 Alliance HPLC system equipped with a Waters 996 photodiode array detector was used for the qualitative analysis of polyphenolics and flavonoids. The mobile phase consisted of a gradient mixture of 0.1% orthophosphoric acid and methanol (gradient method). The column used was an Intersil C_{18} (5 μm, 4.6×250 mm) maintained at a temperature of 25 °C. The flow rate was set at 1 mL min^{-1}, and the detection wavelength was 280 nm. A volume was transferred from a stock solution containing 10 standards (Gallic acid, Rutin, Chlorogenic acid, Catechin, Ellagic acid, Quercetin, Kampeferol, Apigenin, Hesperidin, and Caffeic acid) with conc 1 mg mL^{-1} for each standard, to obtain solutions with conc. of 10 μg mL^{-1} then inject 20 μL. About 20 mg from each extract sample were weighted individually, then they were dissolved in 1 mL water then the solution was vortex followed by sonicated for 20 min. The solution was filtrated through a 0.45 μm PTFE syringe filter and then injected into the HPLC system.

Plant material and experimental design

For the current study, the seeds used are GRObite Desi Tomato Vegetable Seeds, which are indian non-hybrid seeds with a 99% germination rate and drought tolerance. Seeds were first sterilized in a 0.25% sodium hypochlorite solution. After sterilization, an aqueous extract of *N. glauca*, was applied at various concentrations as part of the priming process. The primed seeds were divided into three groups based on the bio-stimulant concentrations: 20%, 50%, and 100%. Priming was carried out for 48 hours with gentle shaking (Moaaz Ali et al., 2020). Following this, the seeds were sown in greenhouse trays filled with a 1:1 mixture of commercial peat and vermiculite. They were subjected to two irrigation conditions: normal conditions using distilled water and salt-stressed conditions using a 100 mM NaCl solution.

For the co-treatment strategy, sterilized seeds directly were sown in the same conditions and irrigated with the corresponding biostimulant concentrations three times per week. For irrigation, 10 mL of distilled water and a salt solution were used for control and salt-treated primed seeds, respectively. For co-treatment, the same volume was used for each biostimulant concentration and a salt solution for control and salt-treated seeds, respectively. The co-treatment involved irrigating the seeds with the biostimulant three times a week. For both strategies, salt treatment was initiated since the beginning of seed sowing.

Two controls are designed: Negative control means no stressed and no primed seeds; positive control means no primed and salt stressed seeds.

Two seeds were sown in each cell of tray with dimension 54D × 28W × 8.7H centimeters (20 cells / tray). Plants were grown in a growth chamber with 26 °C/70% relative humidity and the photoperiod was 16 h daily. For each treatment, 80 seeds were used. The treatments were replicated three times.

After 3 weeks, seedlings were sorted by epicotyl and radicle for determination of fresh weight (FW), dry weight (DW), photosynthetic pigments, malondialdehyde (MDA) and proline content.

Germination parameters. Seeds were considered germinated when the radicle and epicotyl emerged.

According Wu et al., 2019, we determined Final germination percentage (FGP).

1. FGP = NGS/NTS x 100

Also mean germination time (MGT)

2. MGT=Σ (N1T1 + N2T2 + … + NiTi) /Σ (N1 + N2 … + Ni)

Abbreviations used in above equations were:

NGS: number of final germinated seeds (in the end of germination: 6 days); NTS: number of total tested seeds; Ni: number of seeds germinated in the i^{th} time; Ti: time taken for seed germination at i^{th}.

Photosynthetic pigments. The method used to determine leaf chlorophyll content was that of Arnon (1949). UV spectrophotometer (PG instrument T60 UV V United Kingdom) was used to measure the absorbance of each sample at 663 nm and 645 nm. Using the formula provided by MacKinney (1941), the chlorophyll content was computed and expressed in mg per g FW.

Total Chlorophylls (mg L^{-1}) = 20.2×*A*645+8.02×*A*663

A represents the extract's absorbance at the specified wavelength.

Proline content. The leaf proline content was determined using the method described by Bates et al., 1972. Sulpho-salycylic acid is used to extract proline from dry matter samples. A spectrophotometric measurement at 520 nm is performed based on the complexation of proline with Ninhydrin.

Malondialdehyde (MDA) content. The determination of leaf MDA content was carried out utilizing the Thiobarbituric Acid-Reacting Substances (TBARS) method described by Heath & Packer in 1968. MDA was extracted using trichloroacetic acid (TCA: 10%). The MDA concentration in epicotyl samples was measured as nmol g^{-1} FW.

Statistical analysis. The data depicted in the figures are the average of six replicates per treatment with means ± confidence limits at alpha = 0.05 level. To compare and determine significant differences between means, at probability level ≤ 0.05, ANOVA analysis and Tukey's HSD tests were used. SPSS software version 20.1(IBM version 20.0.2004) was used.

RESULTS AND DISCUSSION

Biostimulant effects on germination parameters

In the absence of *N. glauca*-based biostimulant, salinity reduced the final germination percentage (FGP) (Fig. 1, A). Under normal conditions, *N. glauca* extract used in seed priming or as co-treatment had no noticeable effect on FGP. Under saline conditions, all tested doses of the biostimulant improved the FGP. For seed priming, a

20% concentration was the most effective, reducing the FGP decline by 5% compared to untreated and stressed seeds. In the case of co-treated seeds, the 50% and 100% extract doses reduced the FGP decline by 3.6% and 4.5%, respectively, compared to untreated stressed seeds.

Fig. 1, B illustrates the effects of salinity and different doses of biostimulant on mean germination time (MGT). The results indicate that MGT increases with salinity level, suggesting that seed germination occurs at a slower rate. Under normal conditions, the *N. glauca* extract did not have a significant impact, whether used as a priming method or as a co-treatment. However, in saline conditions, all tested doses of the plant extract reduced MGT compared to untreated and stressed seeds, particularly when used as a co-treatment. In priming treatments, a 20% dose was most effective in reducing MGT when compared to untreated and stressed seeds (22%). In co-treatment, a 50% dose was most effective, showing a reduction of 28% relative to untreated and stressed seeds.

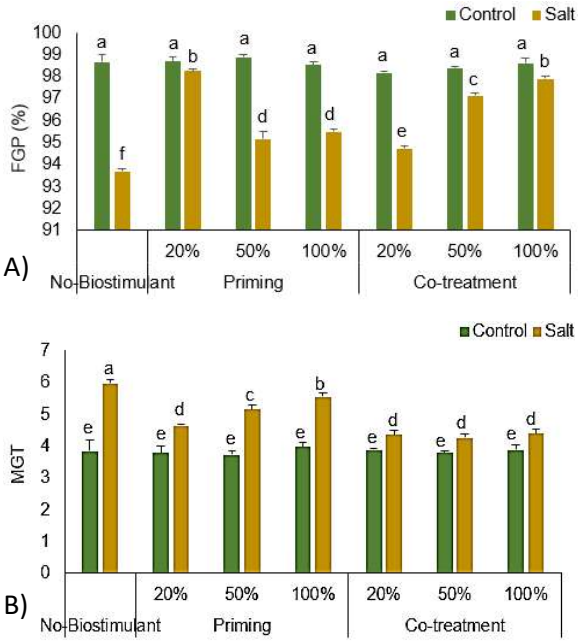

Figure 1. Effect of different concentration of *N. glauca* aqueous extract (20%, 50% and 100%) on Final germination percentage (FGP) (A) and mean germination time (MGT) (B) in normal and salt conditions.

Different treatments were applied: (No-Bio-stimulants: seeds sown without *N. glauca* extract in normal and salt conditions, priming: seeds were pre-treated with different concentrations of aqueous extract of *N, glauca* (20%, 50% and 100%) within 48 h, in normal and salt conditions. Co-treatment: seeds were irrigated with different concentrations of aqueous extract of *N, glauca* (20%, 50% and 100%) in normal and salt conditions. Data are means of six replicates. Comparative lowercase letters (a, b, c, etc.) denote treated and control samples. The Tukey test reveals no significant difference between bars denoted by identical letters with a 5% probability.

In the absence of biostimulant, salinity reduced the FGP and increased MGT (Fig. 1, A and Fig. 1, B). Salt stress adversely affects seed germination and seedling growth through osmotic stress, ion toxicity, and oxidative stress. High salinity decreased germination stimulants like gibberellins, increased abscisic acid level, and altered cell membrane permeability and water behavior within the seed, which can impede germination (Uçarlı, 2020). Moreover, *Rosmarinus officinalis* L. (rosemary) and *Artemisia* L. (wormwood) leaf extracts have been shown to improve seed germination in maize under salinity conditions. This improvement is attributed to the enhancement of the antioxidant system and the maintenance of higher photosynthetic efficiency during salt stress (Panuccio et al., 2018). Additionally, the use of moringa leaf extract for priming wheat seeds demonstrated positive effects on

germination and growth, by promoting efficient nutrient uptake while limiting the accumulation of toxic ions and reactive oxygen species (Ahmed et al., 2021).

Previous studies reported too that several seaweed biostimulants and microalgal extracts had an important germination enhancer effect (Supraja et al., 2020; Hussain et al., 2021; Mzibra et al., 2021). The current plant-based biostimulant showed similar effects to those reported with seaweed extract used for seed priming, which improves germination parameters in tomato plants (Di Stasio et al., 2020).

Bio-stimulant effects on growth parameters

In all tested treatments, salt reduced tomato seedling growth (Fig. 2, A; 2, B; 2, C). In normal conditions, all tested doses of plant extract applied as co-treatment had more improvement effect on seedling growth referring to priming treatment (Fig. 2, C).

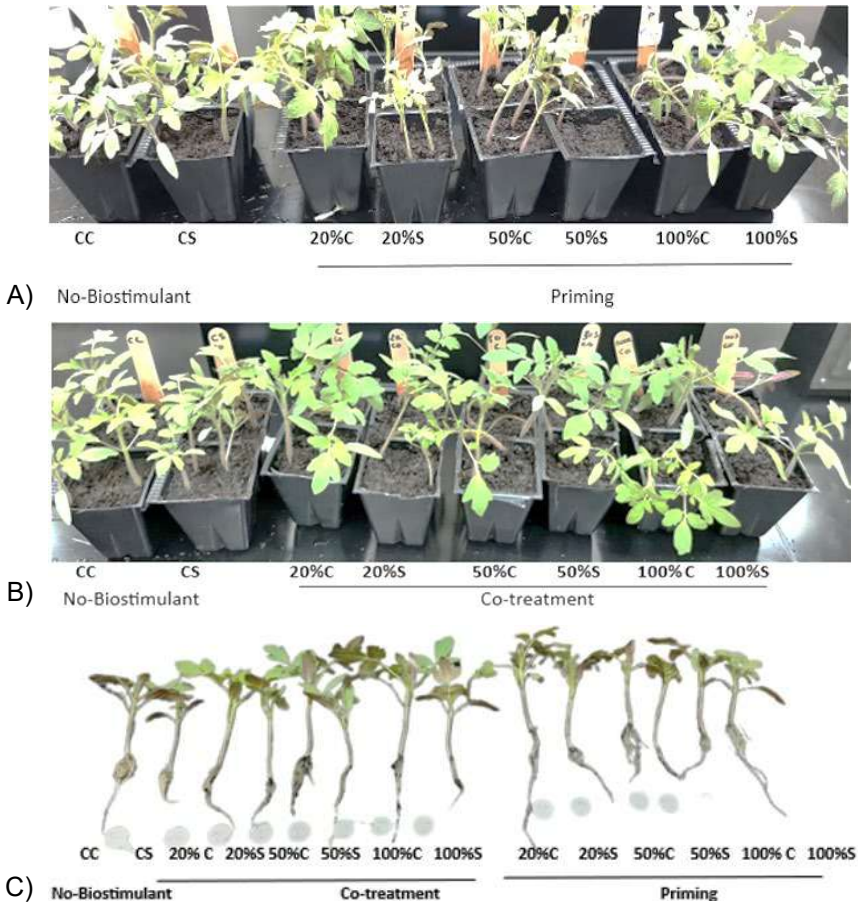

Figure 2. Effect of different concentrations of *N. glauca* extract (20%, 50% and 100%) applied as priming and co-treatment on tomato seedling growth in normal and salt conditions.

(CC: seedlings without extract in normal conditions, CS: seedlings without extract in salt conditions; 20% C and 20%S seedlings treated with 20% of aqueous extract in normal and salt condition as priming and co-treatment; 50% C and 50%S seedlings treated with 50% of aqueous extract in normal and salt condition as priming and co-treatment; 100% C and 100%S seedlings treated with 100% of aqueous extract in normal and salt condition as priming and co-treatment).

Without bio-stimulant, salinity led to a decrease in fresh weight (FW) in the epicotyls (Fig. 3, A). Under normal conditions, co-treatment significantly increased epicotyl FW compared to the control, particularly at the 100% dosage. Seed priming with 20% and 100% plant-based bio-stimulant also improved epicotyl FW, but the effect was less important than that observed with co-treatment.

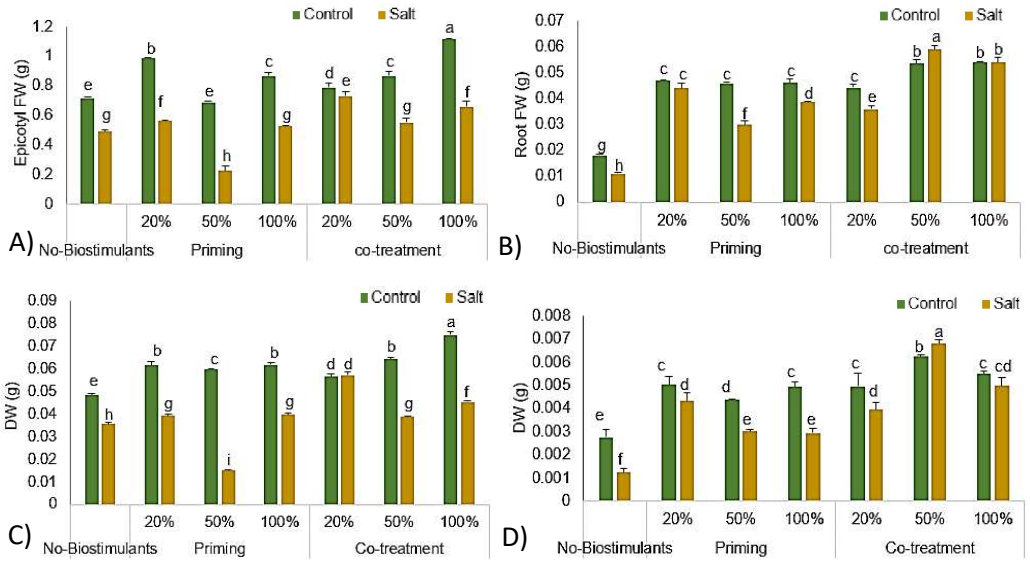

Figure 3. Effect of different concentration of *N. glauca* extract (20%, 50% and 100%) on epicotyl (A) and root (B) fresh weight (FW: g) and epicotyl (C) and root (D) dry weight (DW: g) in normal and salt conditions, as priming and co-treatment.

(CC: seedlings without extract in normal conditions; CS: seedlings without extract in salt conditions; 20% C and 20%S seedlings treated with 20% of aqueous extract in normal and salt condition as priming and co-treatment; 50% C and 50%S seedlings treated with 50% of aqueous extract in normal and salt condition as priming and co-treatment; 100% C and 100%S seedlings treated with 100% of aqueous extract in normal and salt condition as priming and co-treatment). Data are means of six replicates. Comparative lowercase letters (a, b, c, etc.) denote treated and control samples. The Tukey test reveals no significant difference between bars denoted by identical letters with a 5% probability.

Under stress conditions, 20% and 50% doses of plant extract used as a co-treatment significantly increased the fresh weight (FW) of epicotyls. Notably, the 20% dose mitigated the decrease in FW by 49% compared to untreated salt-stressed seedlings. In seedlings issued of primed-seeds, only the 20% dose alleviated the salt-induced reduction in FW (15%), but it was less effective than co-treatment.

In absence of biostimulant, salinity reduced root FW by approximately 40% compared to the control (Fig. 3, B). Under normal conditions, all tested doses used for seed priming or as co-treatments resulted in an increase in root FW, with the 50% and 100% doses as co-treatments showing the most significant effects. In seedlings that were seed-primed, salt stress decreased root FW, with the exception of the 20% dose. When using a co-treatment with 100% of the plant extract, salinity did not have a significant impact on root FW. However, with the 50% dose, salinity actually increased root FW (Fig. 3, B). Overall, under saline conditions, all tested doses helped alleviate the negative effects of salinity on root FW, when applied as co-treatments.

In all examined doses, salinity decreased the epicotyl DW (Fig. 3, C), particularly in untreated stressed seedlings and those derived from seeds that had been primed at 50% extract. Under normal conditions, plant extracts used for seed priming or as co-treatments increased epicotyl DW compared to the control, with the 50% and 100% doses for co-treatment and the 20% dose for seed priming showing the most significant effects. In seedlings that had been seed-primed, salt further reduced epicotyl DW, especially in those from 50% primed seeds (Fig. 3, C). The co-treatment of stressed seedlings was more effective in minimizing the salt decline of epicotyl DW, particularly at the 20% dose.

In roots, in normal conditions, all tested doses used for seed priming or as co-treatment increased DW (Fig. 3, D). Under salinity, seed priming with all tested doses mitigated the salt-induced decrease of root DW. The co-treatment of stressed-seedlings with different extract doses alleviated too the salt-induced decrease in root DW but more effectively compared to seed priming effect.

Chlorophyll content generally correlates positively with photosynthetic capacity and biomass accumulation. A strong relationship has been established between the maximum carboxylation rate in leaves and chlorophyll content across various plant species (Qian et al., 2021). Among the factors influencing the net photosynthetic rate, chlorophyll (Chl) content (particularly Chl b) and stomatal conductance are crucial (Tang et al., 2022). An increase in Chl b content indicates a larger light-harvesting antenna size, thereby enhancing photosynthesis (Tanaka & Tanaka, 2011; Jia et al., 2016). Therefore, studying chlorophyll content is essential for assessing the effects of salinity and the biostimulant alleviation effects.

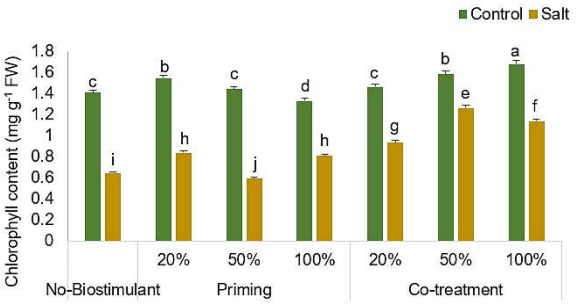

Figure 4. Effect of different concentration of *N. glauca* extract (20%, 50% and 100%) on leaf chlorophyll content (Chl: mg g^{-1} FW) in normal and salt conditions as priming and co-treatment.

(CC: seedlings without extract in normal conditions, CS: seedlings without extract in salt conditions; 20% C and 20%S seedlings treated with 20% of aqueous extract in normal and salt condition as priming and co-treatment; 50% C and 50%S seedlings treated with 50% of aqueous extract in normal and salt condition as priming and co-treatment; 100% C and 100%S seedlings treated with 100% of aqueous extract in normal and salt condition as priming and co-treatment). Data are means of six replicates. Comparative lowercase letters (a, b, c, etc.) denote treated and control samples. The Tukey test reveals no significant difference between bars denoted by identical letters with a 5% probability.

Under normal conditions, both the 50% and 100% extract doses used in co-treatment resulted in a slight increase in Chl content, with an increase of approximately 19% (Fig. 4). The seed priming method led to a 10% increase in Chl content when using a 20% dose. In the absence of plant extract, salt stress reduced Chl content by 45%. However, when comparing untreated seedlings under salt stress to those treated with various extract doses, all treatments, except for the 50% dose used in the priming method, effectively mitigated the negative impact of salt on Chl content. In co-treated, salt-stressed seedlings, Chl content increased by 96% for the 50% dose and by 76% for the 100% dose. These

results demonstrate that all tested doses in the co-treatment method have the potential to mitigate the salt effect on chlorophyll content, as well as on FW and DW.

Several studies reported that salinity affected tomato growth in all stages of plant development (Roșca et al., 2023). Studies done on tomatoes highlighted a negative influence of salinity stress on the physiological parameters such as photosynthetic rate, nitrogen assimilation, transpiration, stomatal conductance, chlorophyll content and mineral uptake (Maeda et al., 2020; Kapadia et al., 2021; Ors et al., 2021; Sassine et al., 2022; Ghorbani et al., 2023).

In literature, several biostimulant types with various doses and method applications were used. Seed priming with *Moringa oleifera* extract, *Garcinia mangostana* and *Cupressus macrocarpa* induced an important salt effect mitigation in *Helianthus annuus*, *Phaseolus vulgaris* and *Cucurbita pepo* (Taha, 2016; Suryaman et al., 2021; El-Sayed et al., 2022). Seed priming induced salt effect mitigation by improving growth parameters photosynthetic capacity, rubisco activities, antioxidant enzyme activities and proline level under salinity (Kapadia et al., 2021).

Current results demonstrated that co-treatment method have more important potential to mitigate the salt adverse effect on tomato growth parameters. Exogenous administration of plant extract boosted the content of photosynthetic pigments during salt stress due to it's abundant in chlorophyll and carotenoids (xanthin, beta-carotene, alpha-carotene, and lutein), which have antioxidant effects (Hasan & sultana, 2018). Sassine et al., reported too, that lithovit-urea and bioumik used as biostimulants, improved salt tolerance in tomato (Sassine et al., 2022).

Recently, Sheteiwy et al. (2021) reported that both seed priming method and foliar application with jasmonic acid showed efficiency in protect early seedlings and alleviate salt stress damage in seedlings in soybean. Similarly, other studies affirmed the effectiveness of both methods in salt stress alleviation (Akram et al., 2020; El-Hawary el al., 2023). Priming seeds enhanced seed imbibition capacity and pre-germinative metabolic processes, leading to improved seedling emergence, growth, vigor, productivity, and adaptability under saline conditions (Biswas et al., 2023). Additionally, seed priming has been shown to reduce the adverse effects of salt-induced oxidative stress on plant biomass and grain yield in wheat cultivars (Ali et al., 2017). The co-treatment helps protect plants against the harmful effects of salinity by enhancing growth, relative water content, membrane stability index, photosynthetic pigment content, soluble sugars, proline, and essential nutrients such as nitrogen (N), phosphorus (P), potassium (K+), calcium (Ca2+), as well as hormones like indole-3-acetic acid, gibberellic acid, and cytokinins. It also improves the K+/Na+ and Ca2+/Na+ ratios and increases the activities of antioxidant enzymes like superoxide dismutase, catalase, and peroxidase, along with contents of glutathione and ascorbate (Rady et al., 2019). Some studies suggest that co-treatment or foliar application is more effective in improving physiological and biochemical parameters, such as ion homeostasis, osmotic adjustment, and antioxidant defense. In contrast, others argue that seed priming is the superior method (Rady et al., 2018; Dustgeer et al., 2021). These conflicting findings indicate that the most effective application method may depend on plant extract type, plant extract concentration, salinity level, and the specific context (Vali et al., 2024). This highlighted the necessity for a more thorough understanding of the underlying mechanisms and their interactions with plant responses under different salinity conditions.

Bio-stimulant effect on plant oxidative status

Malondialdehyde (MDA) is frequently used as a marker for oxidative lipid injury, and its levels fluctuate in response to abiotic stresses (Davey et al., 2005). Under normal conditions, applying different doses of plant extract did not significantly impact the leaf MDA levels (Fig. 5, A). However, in the absence of biostimulant treatment, MDA content doubled under salinity stress. The rise in MDA concentration under salinity was reported in several species such as tomato, in wheat and tunisian squash (Alzahib et al., 2021; Tarchoun et al., 2022; Shahzadi et al., 2024). The increase of MDA content is attributed to increased lipid peroxidation rate caused by salt-induced oxidative stress (Hnilickova et al., 2021).

When exposed to salt stress, all tested doses of *N. glauca* extract effectively reduced the salt-induced increase in MDA levels. Specifically, co-treating seedlings with 50% and 100% doses under salinity conditions resulted in the most significant mitigation of MDA levels. Similar results were revealed when using Moringa leaf extract, Chitosan and seaweed extract to mitigate salt-stress effect in damask rose (Hassan et al., 2020; Elkarmout et al., 2022; Abd-Elkader et al., 2023).

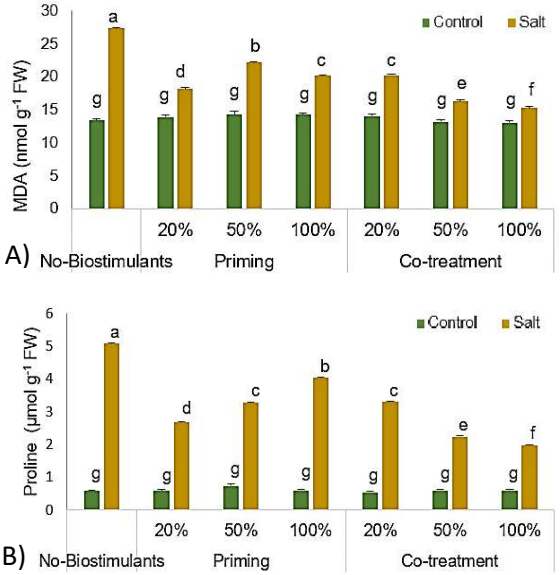

Figure 5. Effect of different concentration of *N. glauca* extract (20%, 50% and 100%) on malondialdehyde (MDA)content (A) and Proline content (B) in leaves of tomato seedlings in normal and salt as priming and co-treatment.

(CC: seedlings without extract in normal conditions, CS: seedlings without extract in salt conditions; 20% C and 20%S seedlings treated with 20% of aqueous extract in normal and salt condition as priming and co-treatment; 50% C and 50%S seedlings treated with 50% of aqueous extract in normal and salt condition as priming and co-treatment; 100% C and 100%S seedlings treated with 100% of aqueous extract in normal and salt condition as priming and co-treatment). Data are means of six replicates. Comparative lowercase letters (a, b, c, etc.) denote treated and control samples. The Tukey test reveals no significant difference between bars denoted by identical letters with a 5% probability.

Plants that face specific abiotic stress conditions often produce proline, which serves several important functions: it acts as an active osmolyte, metal chelator, antioxidant, and signaling molecule (Yaish, 2015). Under normal conditions, the application of various doses of biostimulant, whether through seed priming or co-treatment, did not significantly affect leaf proline levels (Fig. 5, B). However, in the presence of salt stress, Pro levels increased in both control and biostimulant-treated seedlings, with the most significant rise noted in positive control seedlings, which experienced an increase of more than eight-fold. An activation of the defense and adaptation mechanisms associated with increased activity of the antioxidant system, limited the formation of ROS, and suppressed the level of lipid peroxidation. These mechanisms are manifested by a rapid

increase in proline content and osmotic adaptation, which are closely related to salinity tolerance and the ability of the antioxidant system to scavenge free radicals, suppress lipid peroxidation, and promote the accumulation of osmoprotective agents such as proline. Several previous studies reported that proline content was greatly increased in response to the saline environmental conditions (Shin et al., 2020).

Among the seedlings treated with biostimulant, Pro levels tripled in those that received co-treatments with 50% and 100% doses. Therefore, co-treating seedlings with 50% and 100% doses under saline conditions resulted in the most significant reduction of Pro levels referring to positive control.

Several plant-based biostimulants, such as Moringa leaf extract (Howladar, 2014; Rossi et al., 2016; Al-Taisan et al., 2022), have been shown to mitigate the effects of salt stress by reducing ROS damage, as evidenced by lower MDA levels. Pprevious studies on tomato plants demonstrated that biostimulants significantly reduced salt-induced proline accumulation compared to untreated plants (Zuzunaga-Rosas et al., 2023; Zuzunaga-Rosas et al., 2024). A similar effect was observed with a different amino acid-based biostimulant applied to tomatoes (Gil-Ortiz et al., 2023). These findings are consistent with other research (Cristofano et al., 2023) that utilized plant-based biostimulants on lettuce.

Proline is recognized as an excellent biomarker for stress in many crops (Arteaga et al., 2020; Alvarez et al., 2022). Therefore, it can be concluded that these biostimulants help alleviate stress in plants subjected to high salinity conditions. However, this conclusion is not universally applicable, as other studies have reported the opposite effect, with an increase in proline levels in biostimulant-treated plants compared to untreated controls at the same salt concentration. For instance, lettuce plants treated with various plant protein hydrolysates showed heightened proline contents (Zuluaga et al., 2023), and a seaweed extract applied to tomato plants produced similar results (Gil-Ortiz et al., 2023).

Alghamdi et al. (2021) reported varying amounts of alkaloids, steroids, tannins, flavonoid, and saponins were present in the leaf extract of *N. glauca*. theses secondary metabolites had widely reported with potential effect on salt stress mitigation (Ishtiyaq et al., 2021).

Phytochemical characterization of leaf aqueous extract of tested plants

The DPPH radical scavenging assay is a common spectrophotometric method used to measure the antioxidant capacity of beverages, foods, and herbal extracts (Gulcin & Alwasel, 2023). Trolox was used as standard for DPPH assay (Fig. 6). Table 1 illustrated the total phenolic, flavonoid, tannin contents and antioxidant activity. The extract of *N. glauca* demonstrated a significant phenolic content of 97.8 µg eq mg^{-1}. The aqueous extract also showed the presence of flavonoids and tannins, with concentrations of 21.27 µg RE mg^{-1} and 23.25 µg eq mg^{-1}, respectively (Table 1). The results indicated that the *N. glauca* aqueous extract exhibited considerable antioxidant capacity, comparable to various species, such as mistletoe, *Guazuma ulmifolia*, and *Citrus limon* (Rafi et al., 2020; Ehiobu et al., 2021; Khalili et al., 2022). Certain polyphenolic compounds act as scavengers, removing reactive oxygen species (ROS) generated during oxidative bursts. Phenols (ArOH) are recognized for their ability to slow down the oxidation of organic matter by donating a hydrogen atom (from their hydroxyl groups) to chain-carrying ROO• radicals. This process likely involves a coordinated transfer of

hydrogen as a proton along with one electron between the two oxygen atoms, represented as O–H•••O (a proton-coupled electron transfer mechanism) (Foti, 2007).

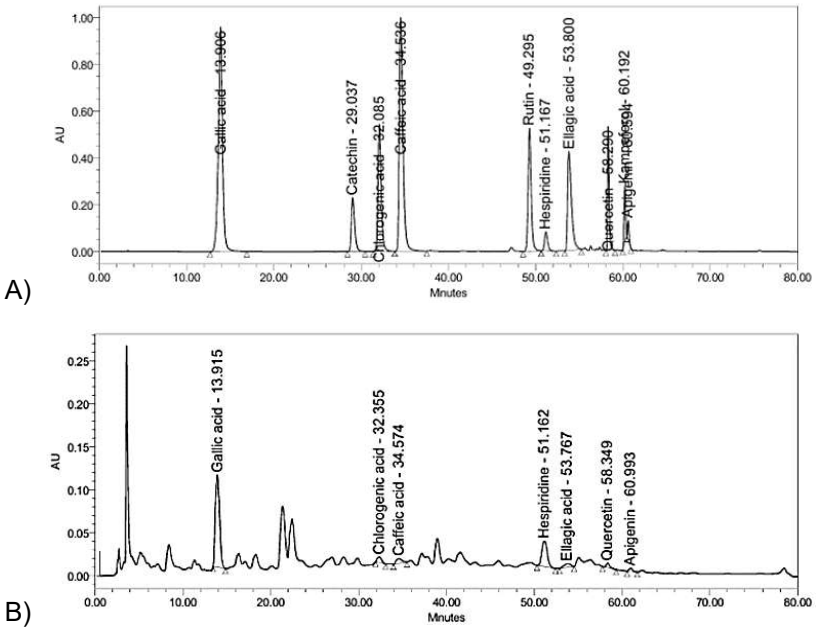

Figure 6. HPLC chromatogram of Mix-standard (A) and *N. glauca* extract (B).

Some of these compounds, such as flavonoids and anthocyanins, have protective properties (Kumar et al., 2023). Polyphenols and flavonoids have been shown to contribute to salt tolerance in various plant species. Kiani et al. (2021) reported that polyphenols play an antioxidant and protective role against oxidative stress induced by salt stress.

Table 1. Total phenolics, total flavonoids, total tannins and Antioxidant Activity (DPPH radical scavenging (ug mL^{-1})) in *N. glauca* aqueous extract. Data are represented as means ± SD

	Total phenolics (µg eq mg^{-1})	Total flavonoids (µg R E mg^{-1})	Total tannins (µg eq mg^{-1})	DPPH free radical scavenging activity: (µg mL^{-1})
N. glauca (Aqueous extract)	97.8 ± 9.38	21.27 ± 1.23	23.25 ± 2.41	162.5 ± 1.02

The significant antioxidant activity and the substantial accumulation of phenolic compounds in the leaves suggest the development of defense-oriented strategies to prevent the buildup of intracellular free radicals generated under salt stress (Kiani et al., 2021). Furthermore, the application of a biostimulant based on tannins affects root architecture and enhances salinity tolerance in tomato plants. The modification of root structure, along with the regulation of genes involved in root growth, abiotic stress responses, and nutrient uptake, indicates that the tannin-based biostimulant may play a crucial role not only in plant nutrition but also in altering morphological traits to improve survival and adaptation to salt stress (Campobenedetto et al., 2021).

For HPLC analysis of aqueous extract, ten standard solutions of phenolics and flavonoids were used (gallic acid, catechin, chlorogenic acid, rutin, ellagic acid, hesperidin, quercetin, kaempferol, apigenin and caffeic acid). The qualitative HPLC analysis of these compounds is summarized in Table 2. The aqueous extract revealed seven components (gallic acid, chlorogenic acid, rutin, ellagic acid, quercetin, apigenin and caffeic acid).

Ozdenefe et al., 2023 reported the presence of antioxidant components in *N. glauca* leaf extract that could protect against the detrimental effects of free radicals. Current result of qualitative HPLC analysis of aqueous extract revealed the presence of several components with antioxidant capacity such us Gallic acid, Chlorogenic acid, Caffeic acid and Ellagic acid (Table 2). This propriety, along with others, have the potential to alleviate the negative effects of salt stress. Current study showed the presence of phenolics, flavonoids and tannins in aqueous extract of *N. glauca* (Table 1). These components play an important role in salt effect mitigation (Ishtiyaq et al., 2023). The aqueous extract revealed seven components (Gallic acid, Chlorogenic acid, Rutin, Ellagic acid, Quercetin,Apigenin and Caffeic acid). Interestingly, these components were related to abiotic stress effect mitigation in several studies. Gallic acid, used as priming agent, is reported efficient in mitigating the adverse effects of salt stress on faba bean at the germination stage (Bouazzi et al., 2024). In rice, Gallic acid reversed salt-induced damage through improving osmotic and ionic homeostasis and upregulating the ROS and MG detoxification system (Rahman et al., 2022). Zhang et al., 2021 found that exogenous phenolics, especially chlorogenic acid, helped lettuce withstand salt stress. Chlorogenic acid mitigate salt adverse effects by triggering significant metabolic changes, enhancing secondary metabolism and the production of small molecules like electron carriers and vitamins and effectively stimulating nitrogen-containing compounds, osmoprotectants, and polyamines. Chlorogenic acid, caffeic acid and apigenin could having a possible role in the cellular defense against oxidative stress (Kianai et al., 2021). Ramzan et al. (2024) reported that using Caffeic acid with had the potential to improve potato growth under salinity stres. Additionally, Muslu, 2024 reported the effectiveness of improving salt stress tolerance by modulating osmolytes accumulation and antioxidant capacity with Rutin. Rutin was also used with Quercetin to enhance antioxidant profile in *Medicago truncatula* (Gaonkaret al., 2024).

Table 2. Qualitative analysis of polyphenolics and flavonoids in aqueous extract of *N. glauca*

Analyte name	*N. glauca* (Aqueous extract)
Gallic Acid	+
Catechin	-
Chlorogenic acid	+
Rutin	+
Ellagic acid	+
Hesperidin	-
Quercetin	+
Kampeferol	-
Apigenin	+
Caffeic acid	+

Note: + present; - Absent.

To summrize, while there is variability in the reported efficiency of biostimulant application methods in the literature, the current study emphasized the greater potential of co-treatment for alleviating salt stress. This effectiveness may be attributed to the continuous exposure to the biostimulant, which is not available to primed seeds. Co-treatment provides a sustained antioxidant effect along with essential nutrients, secondary metabolites, and phytohormones.

Several studies reported that hormonal interactions are essential for enhancing the effectiveness of plant-based biostimulants in salt stress mitigation. Biostimulants like seaweed extracts and humic substances influence hormones such as auxins, cytokinins, and abscisic acid, leading to improved root growth, water-use efficiency, and stress tolerance. For instance, auxin-like compounds in seaweed extracts promote root development for better nutrient absorption in saline conditions (Shukla et al., 2019). Cytokinins delay leaf aging and maintain photosynthesis, while ABA regulates stomatal closure to minimize water loss (Rouphael & Colla, 2018). Additionally, biostimulants reduced ethylene production, preventing premature leaf drop, and enhanced jasmonic and salicylic acid for antioxidant defense (Calvo et al., 2014). Collectively, these interactions boost plant resilience to salt stress, making biostimulants valuable for sustainable agriculture. Recently, Iqbal and Poor, 2024 reported that under stress, plant responses are regulated by defense-related phytohormones such as salicylic acid, jasmonic acid, ethylene and abscisic acid, which act as regulators of tannin production under adverse conditions.

The integration of plant-based biostimulants with conventional fertilization practices optimized their efficiency in mitigating salt stress by enhancing nutrient uptake and improving plant resilience. Biostimulants, such as seaweed extracts and humic acids, work synergistically with fertilizers to improve soil structure, increase nutrient availability, and modulate hormonal pathways, such as auxin and abscisic acid signaling, which are critical for stress adaptation (Rouphael & Colla, 2020). For example, combining biostimulants with nitrogen fertilizers has been shown to enhance root growth and nitrogen-use efficiency under saline conditions, reducing ion toxicity and osmotic stress (Colla et al., 2017). This integrated approach not only improves crop productivity but also reduces fertilizer dependency, promoting sustainable agriculture in salt-affected soils.

CONCLUSIONS

The current study conducted a comprehensive examination of *N. glauca* extract used as a biostimulant to alleviate salt stress effects. The study compared two different application strategies to test their mitigation efficiency at varying doses. Results suggested that co-treatment strategy had more significant salt-effect mitigation. Moreover, the success of these mitigation strategies was critically contingent on the specific doses of the biostimulants, highlighting the importance of precise application in agricultural practices. This result could be related to a continous plant exposure to biostimulant (irrigation three time per week), while, only one time seed exposure was effected for priming strategy.

N. glauca aqueous extract showed promising results in alleviating salt stress. Its effectiveness in alleviationg salinity effects could be attributed to its rich composition of bioactive compounds, including phenolics, flavonoids, and tannins. Chlorogenic acid, caffeic acid, rutin, and apigenin, detected through HPLC analysis of *N. glauca* extract, are recognized for their significant role in cellular defense against oxidative stress. These components bolster the plant's defense mechanisms against salt-induced oxidative stress. Briefly, this study illustrated the potential advantages of *N. glauca* extract used as

biostimulant and underscored the necessity for further research to refine dosage and application methods. Continued exploration of natural plant extracts like *N. glauca* could pave the way for sustainable agricultural practices that enhance crop performance in saline environments.

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