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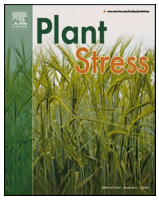

Proline-functionalized graphene oxide nanoparticles (GO-Pro NPs): A new engineered nanoparticle to ameliorate salinity stress on grape (*Vitis vinifera* L. cv Sultana)☆

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ABSTRACT

Salinity stress is of the critical abiotic stress factors in many parts of the world, especially in arid and semi-arid regions and drastically decrease the growth and productivity of the crop. Therefore, in order to maintain the crop productivity and ensure food security in salt-stress suffered regions; in the last decade, novel engineered nanoparticles were synthesized and then assayed for their potential effects concerned with the plant protection against stress conditions. Herewith the present study, we synthesized proline-functionalized graphene oxide nanoparticles (GO-Pro NPs). Then, the interaction effects of either GO-Pro NPs (50 and 100 mg L^{-1}) or graphene oxide nanoparticles (50 and 100 mg L^{-1}) and salinity stress (50 and 100 mM NaCl) on *Vitis vinifera* L. cv Sultana were evaluated by assaying an array of parameters, *viz.* photosynthetic pigments, electrolyte leakage, relative leaf water content, protein, proline, antioxidant defense systems, and oxidative stress markers. Accordingly, salinity critically reduced chlorophyll index and content as well as leaf water content, whilst it caused significant increases in electrolyte leakage, the enzyme activity of superoxide dismutase (SOD) as well as contents of proline, malondialdehyde (MDA) and hydrogen peroxide (H_2O_2). In stress-submitted plants, foliar application of proline-functionalized graphene oxide nanoparticles (100 mM) was effective against oxidative damage with increasing the activity of antioxidant enzymes, proline, and the relative water content of leaves and reducing electrolyte leakage (EL), H_2O_2, and MDA. The current findings revealed that the oxidative stress caused by salinity in Sultana cultivar grapes might be alleviated by foliar application of different levels of proline-functionalized graphene nanoparticles.

1. Introduction

During their life span, plants are constantly exposed to biotic and abiotic stress factors. Among abiotic stress factors, salinity stress is considered to be one of the major environmental constraints, drastically reduce the growth and productivity of plants. The retarded or slowdown in plants is attributed to the accumulation of chlorine and sodium ions, which in turn cause critical alterations in morphological, physiological, and biochemical properties (Mahmoud et al., 2020). Specifically, salinity also imposes osmotic stress in the plant, disruption of photosynthetic activity, breakdown of pigments, and imbalance in water and nutrient uptake. High level of salinity promotes the biosynthesis of reactive oxygen species (ROS), which subsequently induces the onset of oxidative stress. In order to interrupt the cascades of oxidation,

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enhanced activity of antioxidant enzymes and accumulation of proline are of the marked alterations as a response against salinity stress. Consequently, the perturbations in primary metabolism of the plants are manifested as delayed growth and reduced yield (Schneider et al., 2019).

As of the widespread osmo-protectants, the metabolism and oxidation of proline is majorly crucial for plants against stress. Stress-mediated generated ROS modifies the expression of levels of molecules to be channeled to the proline biosynthesis (Wani et al., 2021). Therefore, the changes in proline metabolism, mainly by regulating the activity of enzymes, can improve stress tolerance. For instance, exogenous applications of proline attenuated the adverse effects of salinity stress on plant growth, improved gas exchange parameters, increased soluble protein content, preserved plant water content, reduced Electrolyte leakage and increased the activity of some antioxidant enzymes such as catalase (CAT), SOD, peroxidase (POX) and reduced lipid peroxidation (El Moukhtari et al., 2020).

In the recent decades, nano-engineered conjugated materials have been widely assayed for their potential against stress conditions and consequently, enhanced plant tolerances against stress have been achieved (Singh et al., 2021). Of the relevant materials, graphene, as a carbon-based material, has a wide spectrum of uses due to its exceptional physical and chemical properties, good thermal stability, high electrical conductivity, and mechanical strength (Chen et al., 2018). Graphene oxide is one of the most important members of the graphene family and has unique properties, a two-layer structure, and a high potential for applications in industry, medicine, and medicine. Considering its uses in agriculture, graphene critically increased the growth and yield of leaves, roots, branches, flowers, and fruits (Chakravarty et al., 2015). With respect to the action mechanism, nanomaterials have been reported to enter plant tissues and cells and affect the antioxidant system and cellular metabolism in plants (Gohari et al., 2021a). It is essential to understand the interactions between graphene oxide and plant cells in order to maintain the plant tolerance in stress-affected regions. However, the information linked to the exact *modus operandi* of the nanoparticles is quite limited and for this reason, the relevant researches remain and worth to be investigated. Even not fully-elucidated, applications for nanoparticle graphene in crop promotion as well as pest and insect control have recently received much attention in agriculture (Glomstad et al., 2016). Recently, there have been reports of the use of nanoparticles under salinity stress and increased enzymatic antioxidant properties to withstand stress (Mohammadi et al., 2021; Ganjavi et al., 2021). Wang et al. (2014) reported that exposure to graphene oxide combined with salinity stress caused severe changes in the expression patterns of genes required for root growth and stress tolerance in Arabidopsis. Gao et al. (2020) found that foliar application of graphene oxide at low concentrations significantly reduced the symptoms of cadmium toxicity in lettuce and increased net photosynthesis, stomatal conductance, transpiration rate, and chlorophyll content in plants treated with graphene oxide. In addition, the accumulation of ROS reduced MDA content and the activity of antioxidant enzymes. Park et al. (2020) showed that graphene oxide could be used as a regulatory tool to increase plant growth and stability, especially under stress. Furthermore, Fatehi et al. (2022) confirmed that the GO-Pro NPs could be consider as a potential molecule in order to increase plant tolerance against salinity stress in Moldavian Balm (*Dracocephalum moldavica*).

Grape (*Vitis vinifera* L.; Vitaceae) is native to Mediterranean region but it is cultivated in many parts of the world for fresh consumption and industrial processing due to its high economic and nutritional value. The grape berries and their derivatives, i.e, wine, dried raisins, are characterized with high antioxidant properties due to the phenolic compounds available (Nasser et al., 2020). Iran is one of the most important producers in the world, with an area of 155203 hectares and a production of 1945930 tons (Food and Agriculture Organization of the United Nations Statistics, 2019). As of the most crops, grape is also relatively sensitive plants to salinity stress (Gohari et al., 2021b). Considering the proven protective functions of solo exogenous applications of proline (De Freitas et al., 2019) and graphene oxide (Pérez-Labrada et al., 2019) and GO-Pro NPs (Fatehi et al., 2022) against stress conditions; we, herewith the study, investigated the potential effects of proline-functionalized graphene oxide (GO-Pro NPs) on grape exposed to the salinity stress by estimating an array of parameters such as agronomic, physiological and biochemical attributes of the plant.

2. Materials and methods

2.1. Nanomaterial synthesis and characterization

Graphene oxide (GO) and GO-Pro NPs were synthesized based on our previous published report (Fatehi et al., 2022). Briefly, graphene oxide (GO) was prepared from graphite powder using a modified Hummers strategy (Hummers and Offeman, 1958). The GO–Pro NPs were synthesized in a 50 mL round bottom flask containing 0.2 g of GO, 1.0 g of KOH aqueous solution, 0.8 g of proline. Afterwards, the solution was vigorously stirred for 6 hours and was centrifuged (8000 rpm, 10 min). In order to eliminate unreacted staring materials and contamination, the sample was repeatedly washed with distilled water.

2.2. Plant materials, experiment design and treatments

This experiment was performed on Sultana cultivar grape seedlings (*Vitis vinifera* cv. Sultana) in the research greenhouse of the Faculty of Agriculture, University of Maragheh, Iran. Three-year rooted cutting of grape was obtained from commercial nursery in Maragheh and were planted in 5-liter plastic pots containing coco peat and perlite (1: 1 ratio). The seedlings were left for establishment with supplementation of half strength Hoagland solution (pH = 6-6.5) for one month. Plants with ~8 fully expanded leaves were subjected to non-saline (control) and different levels of salinity (0, 50 and 100 mM) by watering with nutrient solution. Every seven days, complete leaching of the plant growth medium was performed with distilled water to minimize the changes in EC and pH due to salinity. Following the first month after the onset of salinity stress, the particles were foliar-sprayed for three times with an interval of five days. The upper leaf surfaces of control and salt-treated seedlings were sprayed until full wetting. The experiments were performed as a factorial in a completely randomized design with three replicates. Experimental treatments included salinity factor at three levels of NaCl (0, 50, 100 mM), the second factor included foliar spraying of graphene oxide nanoparticles GO NPs (0, 50 and 100 mg L^{-1}), and proline-functionalized graphene oxide nanoparticles (GO-Pro NPs) (0, 50 and 100 mg L^{-1}), and distilled water as a control. Each experiment was carried out using three independent biological replicates. For physiological and biochemical analyses, fully developed leaves were used.

2.3. Chlorophyll index and content of photosynthetic pigments in leaves

A chlorophyll meter (SPAD - 502) was used to measure the greenness of the leaves. Chlorophyll content including chlorophyll a, b, total, and carotenoids were quantified according to the method of Lichtenthaler (1987) by recording the absorbances at 470, 646 and 663 nm (Shimadzu, Model UV 1800, Kyoto, Japan).

2.4. EL and leaf relative water content (RWC)

EL was determined according to the method of Lutts et al. (1996). The RWC was determined according to the method of Sairam et al. (2002).

2.5. Proline and Total soluble protein

Firstly, fresh plant samples (0.5 g) were digested with 10 ml of 3% sulfosalicylic acid. Then, the extracts were centrifuged at 15.000 rpm for 15 min at 4°C. Following the centrifuge, 2 ml of the supernatant were mixed with the addition of 2 ml of ninhydrin acid, and 2 ml of glacial acetic acid. Then, the samples were placed in a hot water bath for 1 h at a temperature of 90°C. The samples were then transferred to an ice-containing medium. After cooling, 4 ml of toluene was added to each sample and vortexed. For proline content, absorbance of the samples was recorded at 520 nm (Bates et al., 1973). The total soluble protein content of samples were quantified according to the method of Bradford (1976).

2.6. Estimation of antioxidant enzymes activity

Ascorbate peroxidase (APX) activity was measured by the method (Nakano and Asada, 1987). Briefly, the reaction mixture contained 2550 µl of 0.5 mM ascorbate dissolved in 100 mM potassium phosphate buffer, 450 µl of 2 mM H_2O_2, and 450 µl of double-distilled water. APX activity was measured at 290 nm using a spectrophotometer. The estimation of SOD activities was based on the inhibition of photochemical reduction of nitroblo tetrazolium (NBT). In this method, the reaction mixture contained 50 mM potassium phosphate buffer (pH = 7), 13 mM methionine, 75 µM nitrobltetrazolium, four µM riboflavin, and 200 µl of plant extract. The reaction was started by adding riboflavin and placing the tubes under two 15-watt fluorescent lamps for 15 minutes. Test tubes containing reaction mixtures that were not affected by the light source were considered as control (Giannopolitis and Ries, 1977).

2.7. Estimation of malondialdehyde and hydrogen peroxide content

MDA content was quantified according to the method of thiobarbituric acid (TBA) method (Qin et al., 2018a, 2018b). Briefly, fresh leaves (0.5 g) were homogenized using 5 ml of 5% trichloroacetic acid (TCA). The homogenates were centrifuged at 10.000 rpm for 10 min. Two ml of the mixture (10% TBA with 0.67% TCA) was added to the supernatant. The test tubes were placed in boiling water for 30 min. Then, the tubes were quickly transferred to ice. The samples were centrifuged again at 10.000 rpm for 5 min. Ultimately, the absorbances of the samples were recorded at 450, 532, and 600 nm. Finally, MDA concentration was quantified using the following formula: MDA (µmol/g FW) =6.45 (OD_{532}-OD_{600})–0.56 OD_{450}

For determination of peroxide H_2O_2 content, fresh leaf samples (0.5 g) were firstly extracted with 5 ml of 0.1% trichloroacetic acid solution. The extracts were then centrifuged at 12.000 rpm for 15 min at 4°C. Following the centrifuge, a mixture containing 0.5 ml of supernatant, 0.5 ml of 10 mM potassium phosphate buffer (pH=7) and 1 mL of 1 M potassium iodide was prepared and its absorbances were recorder at 390 nm. The content was quantified with a standard calibration curve of different concentrations of H_2O_2 (Loreto and Velikova, 2001).

2.8. Statistical analysis

Data were subjected to analysis of variance (ANOVA) (SAS, version 9.4), following Duncan Multiple Range post-hoc at $p<0.05$ for comparison. The experiments were carried out with three replicates and each replicate corresponded to the pools of three biological replicates.

3. Results and discussion

3.1. Synthesis and characterization of GO-Pro NPs

GO-Pro NPs were synthesized in two steps. In the first step, graphite powder was used as starting material to synthesis of GO through well-known modified Hummers method (Eftekhari et al., 2018). In the second step, GO surface was modified by proline in basic medium. From FT-IR analysis, characteristic bonds at 1311, 1626 and 3326 cm^{-1} corresponding to C-N, C=O and N-H, respectively, confirmed the formation of chemical bond between GO and Pro (Fig. 1A). Moreover, SEM image of GO-Pro NPs showed a layer-like morphology (Fig. 1B)

3.1. Chlorophyll index and photosynthetic pigments (Chl a, b, total Chl, and carotenoids)

Salinity stress reduced the chlorophyll index so that severe salinity (100 mM NaCl) decreased in the chlorophyll index compared to the control. On the other hand, foliar application of GO NPs (100 mM) at 50 mM salinity enhanced the chlorophyll index in compared to non-salinity condition (Fig. 2).

Furthermore, the Chl *a* and *b* content in severe salinity stress (100 mM) reduced by 42.2% and 52.1%, respectively, in comparison to the control (Fig. 3A and B). The foliar application of GO-Pro NPs (50 and 100 mg L^{-1}), significantly increased the Chl *a* and *b* in non-stress conditions in comparison to the control. Also, total chlorophyll content decreased in favor of increasing salinity in comparison to the control (44.4%). Plant treatment with 100 mg L^{-1} of GO-Pro NPs enhanced the total chlorophyll content by 15.8% (Fig. 3A–F). The highest content of carotenoids was observed in the treatment of GO-Pro NPs (100 mg L^{-1}), which increased the content by 19.2% in comparison to the control (Fig. 4).

Decreased chlorophyll content in plants under salinity is considered as a clear sign of oxidative stress, which is due to the activation of chlorophyllase enzyme to be active in the destruction and inhibition of

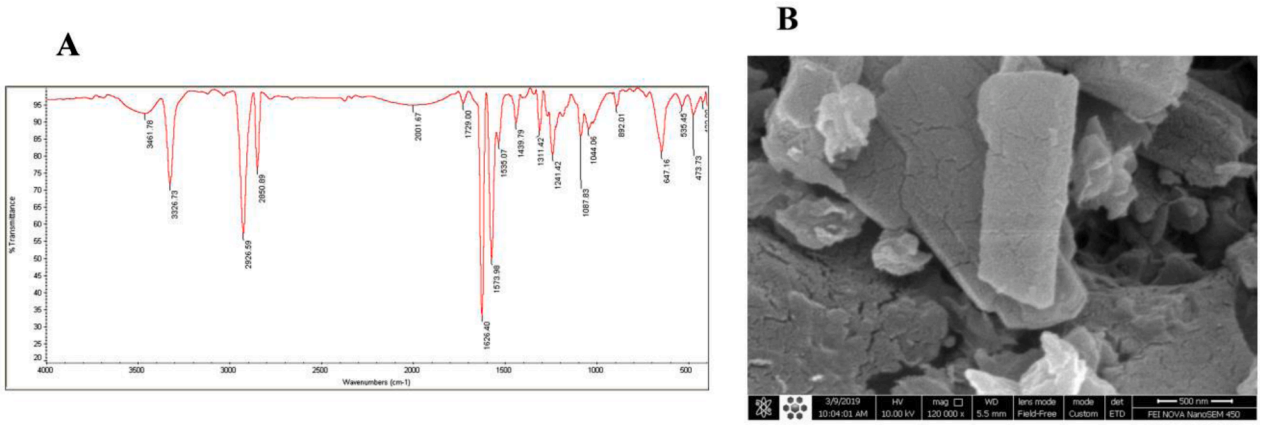

Fig. 1. FTIR curve (A) and SEM image of GO-Pro NPs (B).

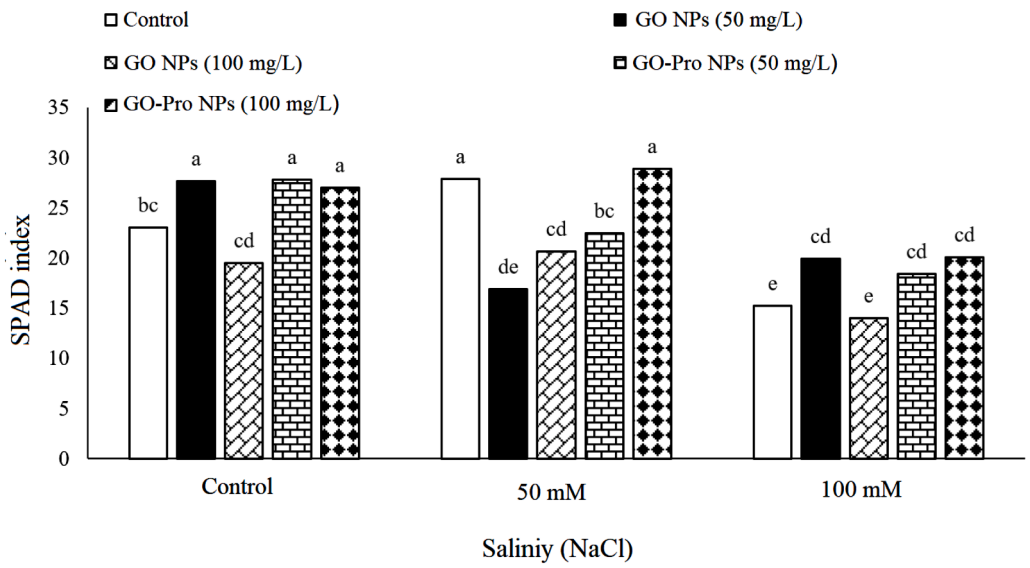

Fig 2. Effect of foliar application of graphene oxide nanoparticles (GO NPs: 50 and 100 mg L^{-1}), and proline-functionalized graphene oxide nanoparticles (GO–Pro NPs: 50 and 100 mg L^{-1}) on chlorophyll index of grape (*Vitis vinifera* L. cv Sultana) under non-stress (0 mM NaCl) and salt stress (50 and 100 mM NaCl) conditions. Values in columns with different letters are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

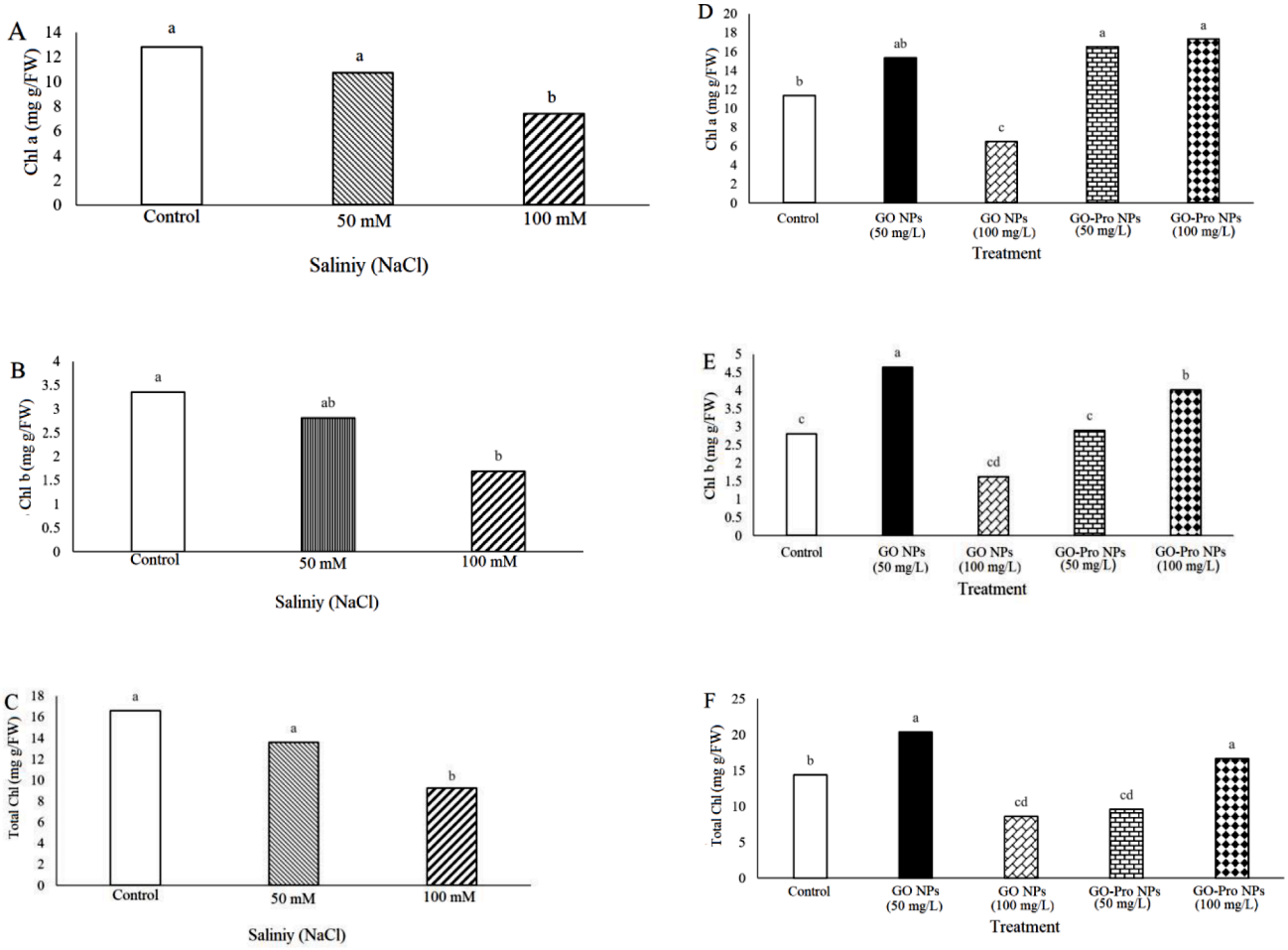

Fig 3. Effect of foliar application of graphene oxide nanoparticles (GO NPs: 50 and 100 mg L^{-1}), and proline-functionalized graphene oxide nanoparticles (GO–Pro NPs: 50 and 100 mg L^{-1}) on photosynthesis pigments (Chl a, b and total Chl) of grape (*Vitis vinifera* L. cv Sultana) under non-stress (0 mM NaCl) and salt stress (50 and 100 mM NaCl) conditions. Values in columns with different letters are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

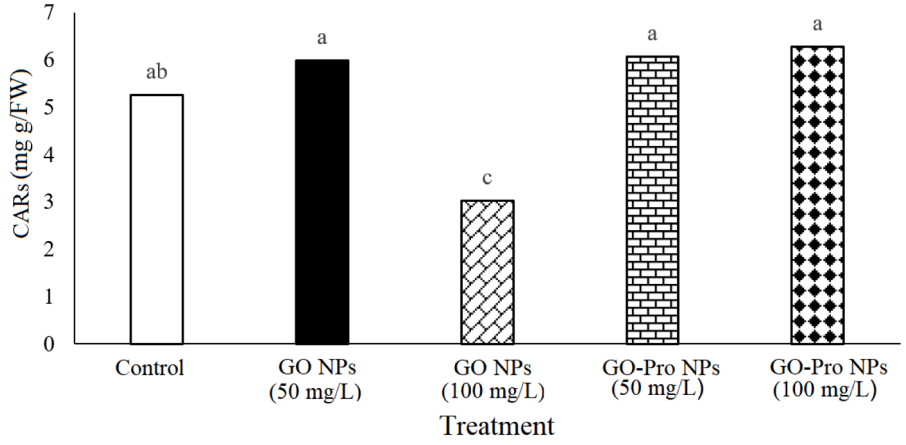

Fig 4. Effect of foliar application of graphene oxide nanoparticles (GO NPs: 50 and 100 mg L^{-1}), and proline-functionalized graphene oxide nanoparticles (GO–Pro NPs: 50 and 100 mg L^{-1}) on carotenoids (CARs) of grape (*Vitis vinifera* L. cv Sultana) under non-stress (0 mM NaCl) and salt stress (50 and 100 mM NaCl) conditions. Values in columns with different letters are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

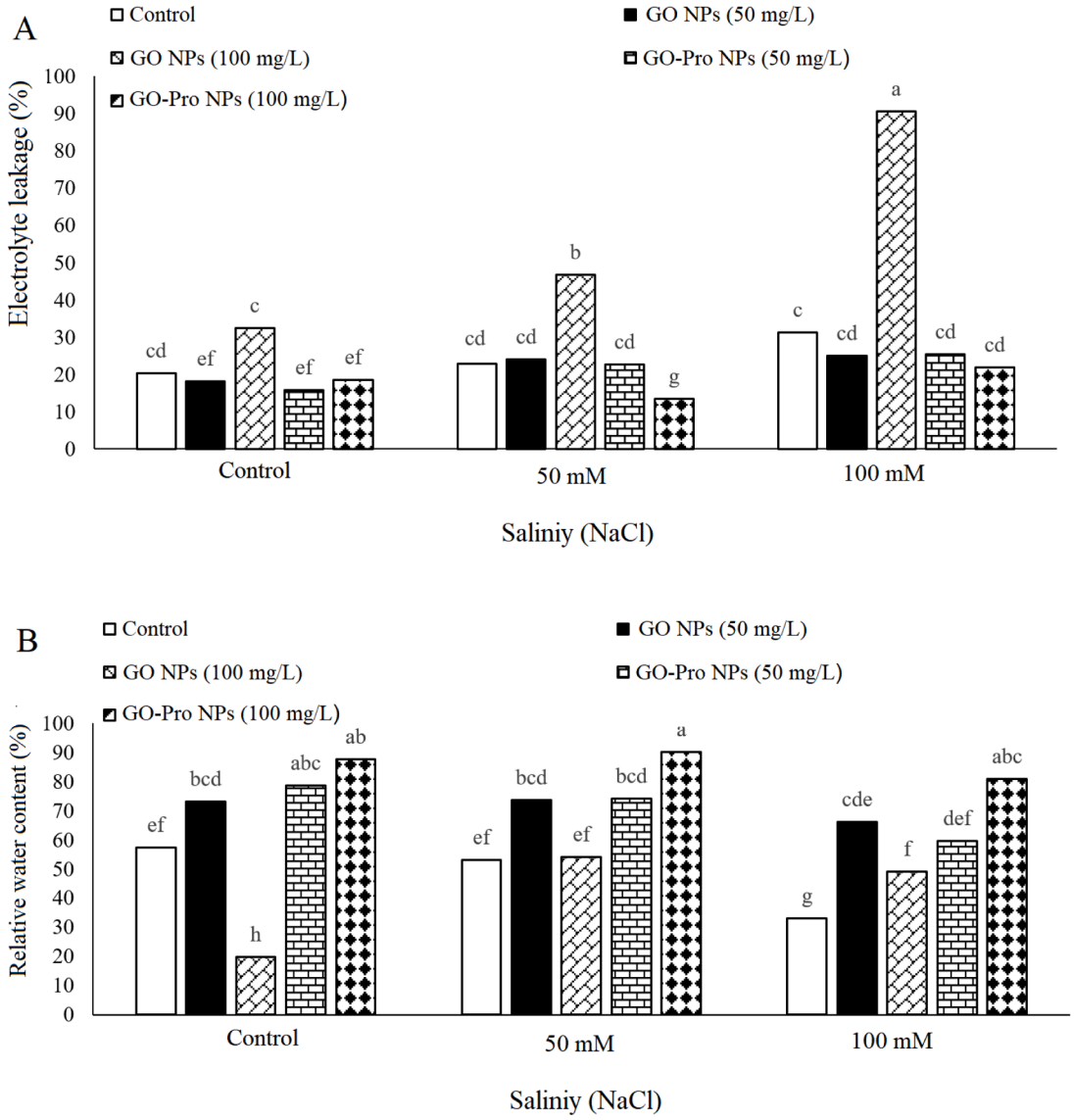

Fig 5. Effect of foliar application of graphene oxide nanoparticles (GO NPs: 50 and 100 mg L^{-1}), and proline-functionalized graphene oxide nanoparticles (GO–Pro NPs: 50 and 100 mg L^{-1}) on electrolyte leakage (EL: A) and relative leaf water content (RWC: B) of grape (*Vitis vinifera* L. cv Sultana) under non-stress (0 mM NaCl) and salt stress (50 and 100 mM NaCl) conditions. Values in columns with different letters are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

chlorophyll biosynthesis (Anower et al., 2013; Saddiq et al., 2021). Siddiqui et al. (2019) reported that low concentrations of graphene oxide elevated the content of chlorophyll, carotenoids, and plant growth. Since proline acts as a protective molecule between different compatible solvents and improves plant conditions in a stressful environment, it also stabilizes chloroplast by stabilizing electrons through transferring electrons in photosystem II, stabilizing membranes and enzymes (Sharma et al., 2012). In addition, exogenous proline treatment due to proline chloroplast stabilization, structure maintenance, and activation of chlorophyll biosynthetic enzymes is likely to reduce membrane damage and plant growth under stress by maintaining chlorophyll pigments (Shafiq et al., 2014; Stefanov et al., 2021).

3.2. EL and RWC content

EL occurs as a result of lost membrane integrity maintenance (Hnilickoya et al., 2019). As expected, increasing of NaCl concentration in growth medium led to increased EL in all treated plant in comparison to the control. The level of electrolyte leakage was buffered with both concentrations of GO-Pro NPs for plants subjected to salinity stress (Fig. 5A). Plasma membranes are the main site of sodium chloride ion damage. Therefore, EL from plasma membranes has been reported as one of the most important selection criteria for the identification of plants resistant to salinity stress (Ashraf and Ali, 2008). In this regard, we can suggest that increasing level of salinity induced a higher level of EL, which might be a critical sign indicating the disturbance of plasma membrane because of the increases of sodium chloride levels in the growing media. In addition to high level of salinity, the high levels of graphene oxide also disrupt the membrane integrity, which in turn result in critical changes in membrane potential, membrane energy transfer system, and ATP levels inside and outside the cell (Lyon and Alvarez, 2008).

Corresponding to severity of salinity, RWC critically decreased by 42.1% in comparison to the control. On the other hand, foliar application of GO NPs and GO-Pro NPs increased 57.4% and 41.2% of the RWC in comparison to the control, respectively (Fig. 5B). Relative water status of the plants is also crucial indicator in stress researches, being linked to the balance between water uptake and transpiration. Along with the accumulation of sodium chloride around root system of the plant, the uptake and absorption of water are critically restricted and the restriction is translated to the delayed and retarded growth and performance through significant decreases decrease in photosynthesis (Ansari et al., 2019) and leaf area, and increases in leaf membrane damage (De Freitas et al., 2019).

On the other hand, nanomaterials, i.e, carbon nanotubes, can regulate cell division and plant growth by a unique molecular mechanism involved in the activation of water channels (aquaporins) and the major regulators of genes in cell division and proliferation (Khodakovskaya

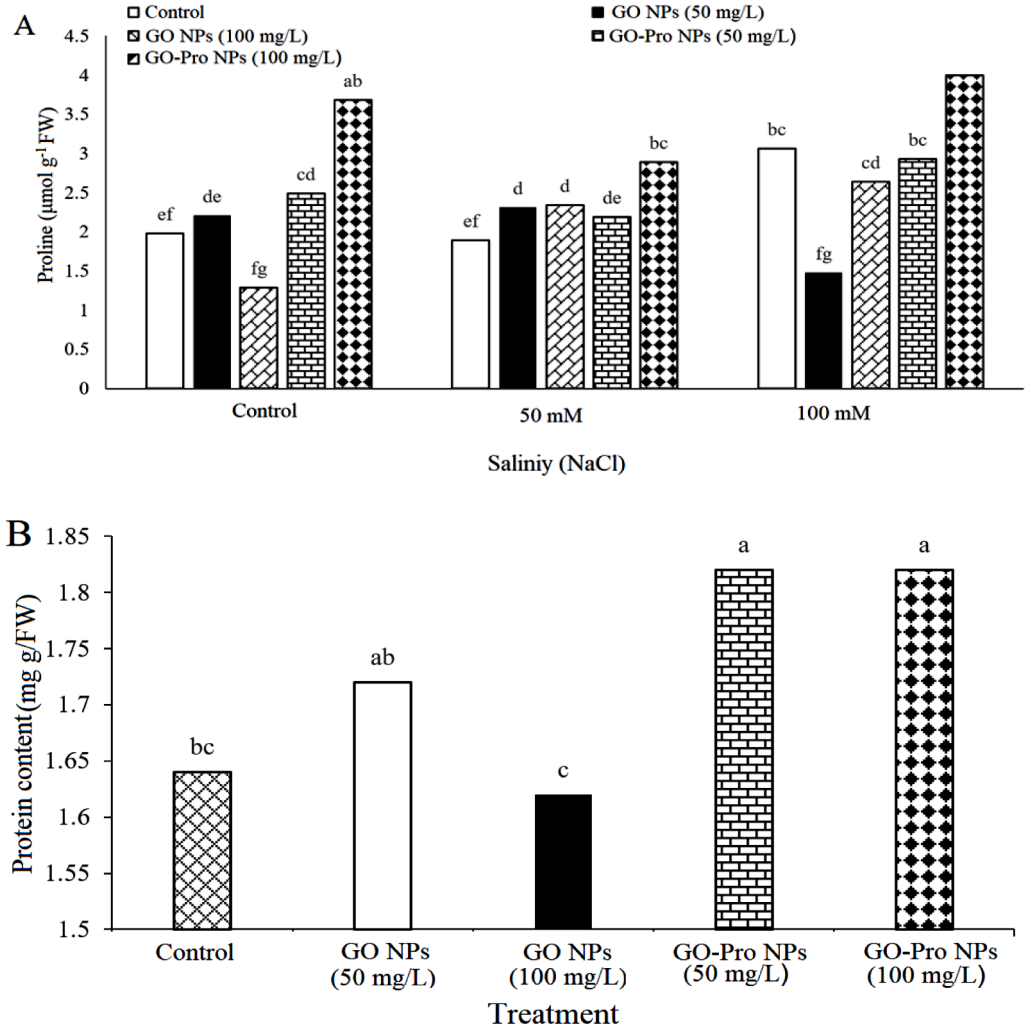

Fig 6. Effect of foliar application of graphene oxide nanoparticles (GO NPs: 50 and 100 mg L^{-1}), and proline-functionalized graphene oxide nanoparticles (GO–Pro NPs: 50 and 100 mg L^{-1}) on proline (A) and protein content (B) of grape (*Vitis vinifera* L. cv Sultana) under non-stress (0 mM NaCl) and salt stress (50 and 100 mM NaCl) conditions. Values in columns with different letters are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

et al., 2012). Also, Zhao et al. (2020) reported that the RWC decreased with increasing drought stress but was higher in GO NPs treated roots, stems, and leaves than in controls. With the externally supplied proline (25 and 50 mM), higher RWC was observed, in relation to the groups without proline supplementation (Ben Ahmed et al., 2011). Another possibility is that maintaining the optimal amount of water under osmotic stress may be attribute to the regulator of root aquaporin gene expression in response to exogenous proline (Moukhtari et al., 2020).

3.3. Proline content and total soluble protein

As expected, salinity stress caused significantly increased proline content. Specifically, foliar application of GO-Pro NPs (100 mg L^{-1}) at 100 mM salinity level increased the content of proline by 102.0% in comparison to the control. However, the highest proline content was achieved in plant treated with 100 mg L^{-1} of GO-Pro NPs (Fig. 6A). The critical roles of proline, as a phytoprotectant, have been well reported against environmental stress factors, in general and salinity stress, in particular, being linked to the osmotic regulation of materials, maintenance of membrane integrity, removal and inhibition of ROS, and improvement of antioxidant capacity under osmotic stress (Dong et al., 2014). In this regard, Murkute et al. (2010) stated that one of the reasons for the high tolerance could be due to the higher accumulation of proline through alleviating the damage imposed by osmotic stress and sodium ion chloride. Safikhan et al. (2018) noted that the use of GO increased the proline content of Milk thistle (*Silybum marianum* L.). Also, uses of graphene increased proline content in plants exposed salinity stress (Zhao et al., 2020).

Regarding protein content, application of GO-Pro NPs (100 mg L^{-1}) increased the total protein in comparison to control. On the other hand, the lower content was noted with 100 mg L^{-1} of GO NPs treatment (Fig. 6B). The lowest protein content was seen at 100 mg of GO NPs treatment, which might show the toxic effect of high concentration of GO NPs. The use of nanoparticles in tomato plants increased protein content by activating transcription or translation processes (Faizan and Hayat, 2019). Also, exogenous proline treatment leads to the stability of the 3D structure of proteins, protection of cell organs and membranes against lipid peroxidation, and despite sufficient energy on plant growth

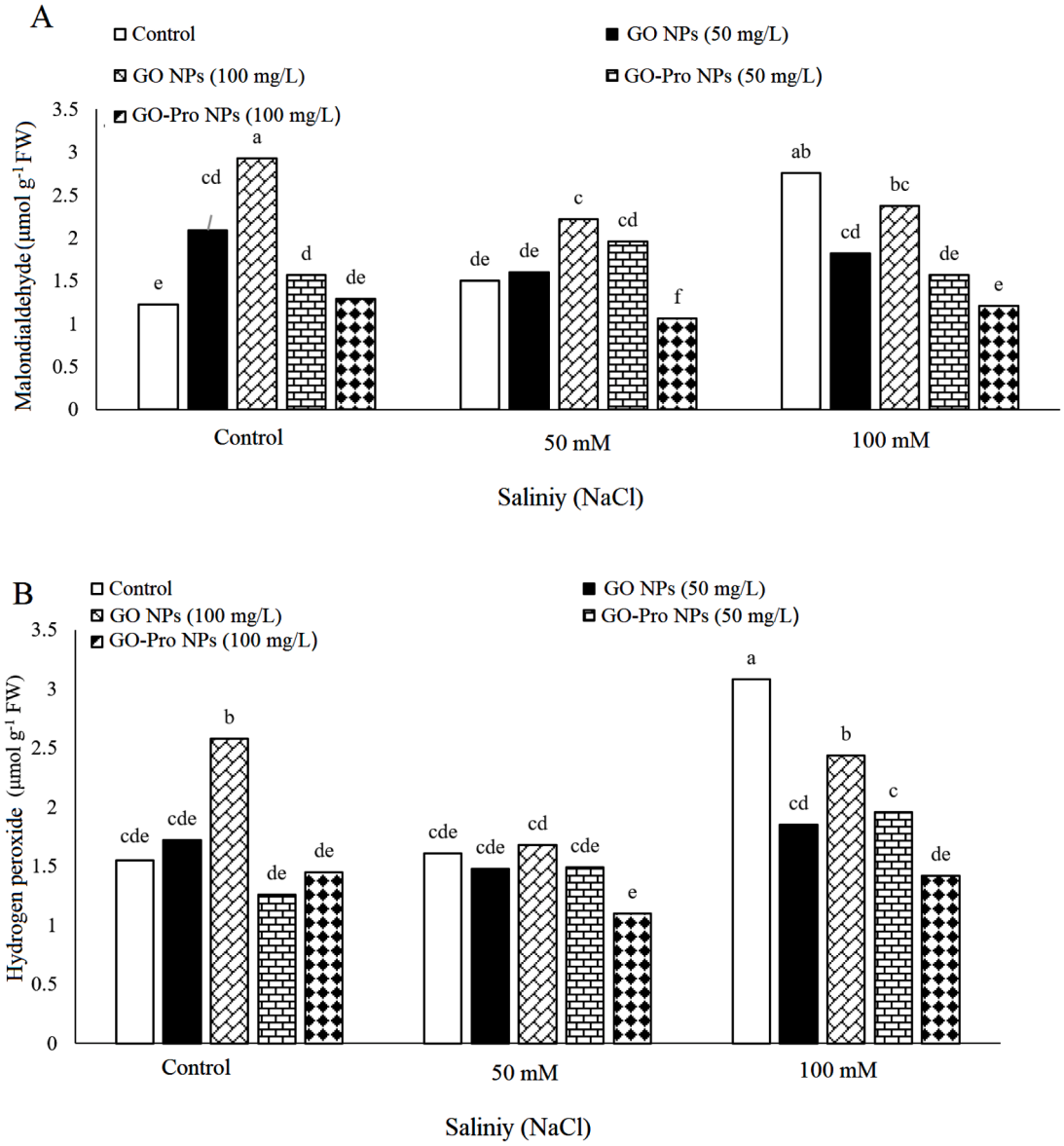

Fig 7. Effect of foliar application of graphene oxide nanoparticles (GO NPs: 50 and 100 mg L^{-1}), and proline-functionalized graphene oxide nanoparticles (GO–Pro NPs: 50 and 100 mg L^{-1}) on malondialdehyde (MDA: A) and hydrogen peroxide (H_2O_2: B) content of grape (*Vitis vinifera* L. cv Sultana) under non-stress (0 mM NaCl) and salt stress (50 and 100 mM NaCl) conditions. Values in columns with different letters are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

and survival, increases the plant's ability to overcome stress (Ashraf and Foolad, 2007). Application of external proline, in addition to the dry weight of leaves and roots, increased protein content in maize plants under salinity stress (de Freitas et al., 2018).

3.4. MDA and H_2O_2 content

MDA and H_2O_2 increased under salinity stress and decreased with the application of proline-functionalized graphene oxide nanoparticles under severe stress. Following the use of the highest concentration of proline-functionalized graphene oxide for salinity levels of 50 mM, the production of MDA dropped by 13.1% in comparison to the control. The highest production of MDA was observed at the level of 100 mM graphene oxide. The lowest was observed at the application of 100 mM proline-functionalized graphene foliar application for plants under 50 mM salinity stress (Fig. 7A). Accumulation of H_2O_2 in grape seed leaves under stress caused by 100 mM sodium chloride concentration had a surge at 98.7% once compared to seedlings without foliar application. The present findings indicate that 100 mM sodium chloride had adverse effects on grape seedlings under natural conditions. The accumulation of H_2O_2 decreased significantly with increasing concentrations of proline-functionalized graphene under salinity stress (Fig. 7B).

Membrane integrity and stability are important for maintaining normal physiological activity in plants. Excessive accumulation of Na^+ can alter the initial balance of free radicals in cells, thus causing membrane lipid peroxidation, which results in decreased membrane fluidity and increased permeability (Ahmad et al., 2014). ROS production increases under stress conditions, ROS reacts with unsaturated fatty acids, lipid hydroperoxides are formed, and membrane permeability is reduced. This action leads to an increase in the content of H_2O_2, electrolyte leakage, and MDA (Alzahrani et al., 2019). Probably the main cause of severe damage to the cell membrane is the production of superoxide radicals, hydroxyl radicals, and H_2O_2, which ultimately leads to the peroxidation of unsaturated fatty acids in the cell membrane.

It has been reported that under stress conditions, exposure to high concentrations of graphene oxide caused more severe effects on plants (*Arabidopsis thaliana*) and altered graphene oxide transfer patterns, leading to an increase in MDA content in Arabidopsis seedlings (Wang et al., 2014). A study by Zhao et al. (2020) also shows that the amount of H_2O_2 in GO-treated leaves was lower in comparison to the control. Proline acts as a compatible solution, osmotic protector, and as hydroxyl radical scavenger. Therefore, an increase in SOD, CAT, and POX activities under salinity stress might be linked to the exogenously applied proline (Wani et al., 2016). Exogenous proline suppresses H_2O_2

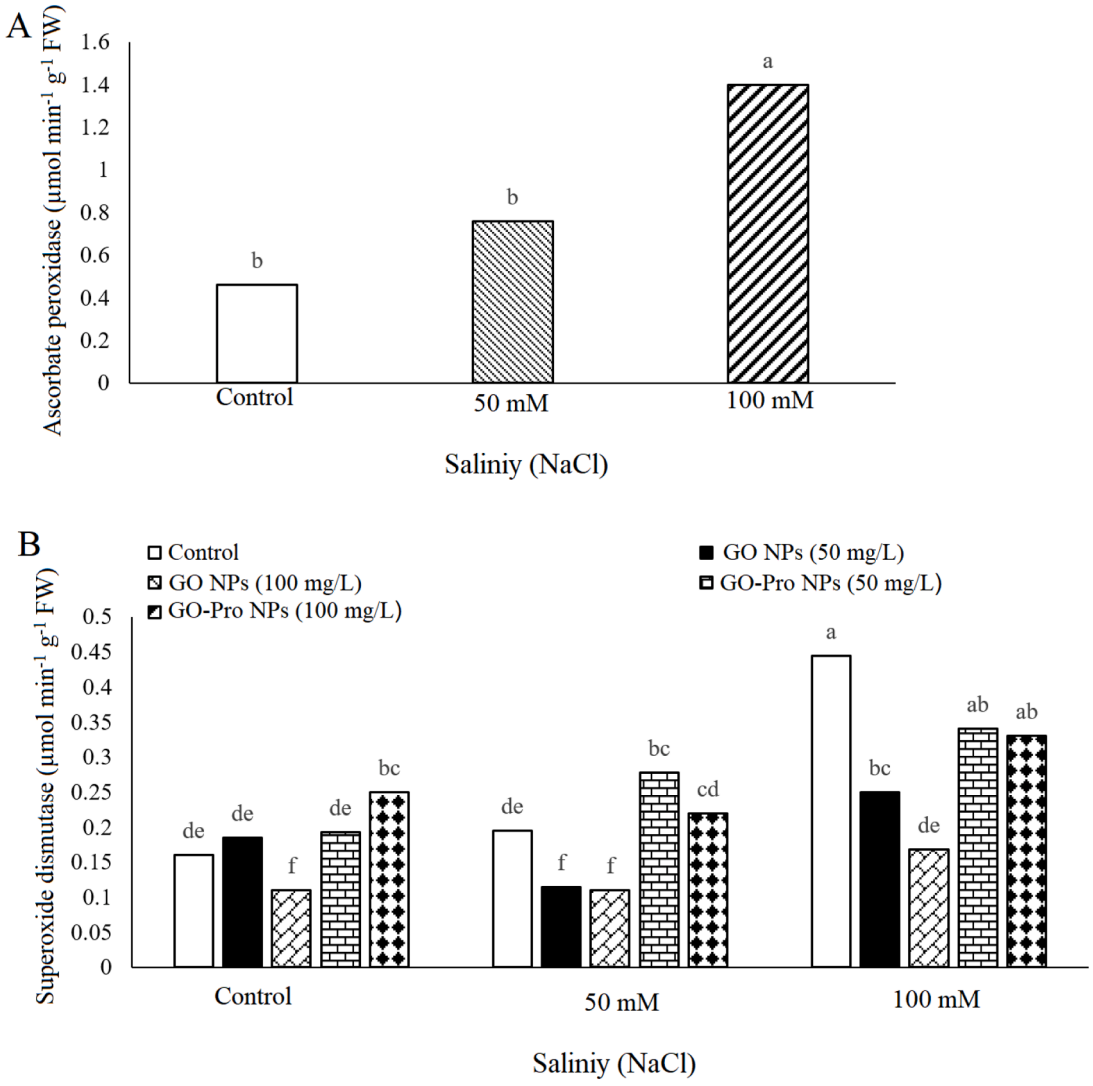

Fig 8. Effect of foliar application of graphene oxide nanoparticles (GO NPs: 50 and 100 mg L^{-1}), and proline-functionalized graphene oxide nanoparticles (GO–Pro NPs: 50 and 100 mg L^{-1}) on ascorbate peroxidase (APX: A) and superoxide dismutase (SOD: B) of grape (*Vitis vinifera* L. cv Sultana) under non-stress (0 mM NaCl) and salt stress (50 and 100 mM NaCl) conditions. Values in columns with different letters are significantly different at $p \leq 0.05$ (Duncan's multiple range test).

accumulation with increased CAT activity under salinity stress. Therefore, it is possible that by affecting the activity of ascorbate peroxidase and catalase, proline-functionalized graphene oxide reduces the production of hydrogen peroxide to reduce oxidative stress damage in grape seedling leaf cells. In addition, exogenous proline slowed down the negative effects of salinity stress on plant growth (Wani et al., 2019), improved gas exchange parameters, expressed proteins against stress, reduced lipid peroxidation, and increased the activity of antioxidant enzymes.

3.5. The activityes of APX and SOD enzymes

During the salt stress condition, a significant increase was observed in the activity of enzymes APX and SOD as antioxidant enzyme (Fig. 8A and B). The activity of the APX enzyme at a concentration of 100 mM of proline-functionalized graphene oxide was about 94.4% once compared to the control plant. However, an 80.3% decrease in APX activity in 100 mM graphene oxide treatment in comparison to the control. Those findings suggest that high concentration of graphene causes critical toxicity. Increased enzyme activity (SOD) in 50 mM proline-functionalized graphene oxide treatment under 50- and 100 mM salinity stress increased by 73.7 and 112.5%, and in 100 mM proline-functionalized graphene oxide treatment under 50- and 100-mM salinity stress increased by 37.5% and 106.2% in comparison to control (Fig. 7B).

ROS production increases when plants were exposed to bio- and abiotic stresses. Plant defense systems can help maintain low ROS levels and prevent membrane lipid peroxidation during stress. In this context, enzymes such as SOD, POD, CAT, and APX are of the vital components related to the ROS maintenance (Gao et al., 2020). Therefore, enhanced activities of the relevant enzymes might contribute to the tolerance of the plants against salinity stress. Considering efficiency of graphene, low concentration graphene oxide nanotubes improved the antioxidant system, which in turn increased the stability and productivity of the plant (Faizan et al., 2019). Zhao et al. (2020) stated that GO treatment leads to a significant increase in enzymatic activity of antioxidants under stress. Therefore, it can be suggested that GO can reduce the accumulation of ROS (Yang et al., 2017). On the other hand, the researchers reported that when proline is exogenously applied in a culture medium under stress, the activity of antioxidant enzymes increases significantly reducing the side effects of oxidative stress consequently (Nakhaie et al., 2022).

4. Conclusion

Salinity is one of crucial factors to impose adverse effects on plant metabolism. Producing active oxygen species damages cell components while at the same time induces chlorophyll degradation, peroxidation of membrane lipids, reduced fluidity, membrane selectivity and processes, and ultimately reduced growth and yield. In this study, the use of two different concentrations of proline-functionalized graphene nanoparticles improved photosynthetic parameters, the activity of APX, SOD, and proline enzymes, together with modifying the salinity stress toxic effects through minimizing the production of certain chemical compounds such as MDA and H_2O_2. Comparing both concentrations of proline-functionalized graphene nanoparticles showed that the highest concentration had the most positive impact in reducing H_2O_2, MDA while increasing proline content, APX and SOD activity, total soluble protein content, and relative cell water content. The beneficial effects of GO-Pro NPs on grape seedling growth performance under different salinity levels were attributed to (1) protection of photosynthetic pigments for enhancing photosynthetic capacity, (2) accumulation of proline and total soluble carbohydrates for osmoprotection, and (3) activation of antioxidant system for efficient ROS homeostasis for improvement of root biomass and maintenance of proper osmotic status of the cells. Our results showed that the foliar spraying of proline-functionalized graphene oxide nanoparticles had positive effects on reducing salinity stress.

Declaration of Competing Interest

The authors declare no competing interest.

Data availability

Data will be made available on request.

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References

Ahmad, P., Ozturk, M., Sharma, S., Gucel, S., 2014. Effect of sodium carbonate-induced salinity–alkalinity on some key osmoprotectants, protein profile, antioxidant enzymes, and lipid peroxidation in two mulberry (*Morus alba* L.) cultivars. J. Plant Interact. 9 (1), 460–467.

Alzahrani, S.M., Alaraidh, I.A., Migdadi, H., Alghamdi, S., Khan, M.A., Ahmad, P.A.R., 2019. Physiological, biochemical, and antioxidant properties of two genotypes of *Vicia faba* grown under salinity stress. Pak. J. Bot. 51 (3), 786–798.

Anower, M.R., Mott, I.W., Peel, M.D., Wu, Y., 2013. Characterization of physiological responses of two alfalfa half-sib families with improved salt tolerance. Plant Physiol. Biochem. 71, 103–111.

Ansari, M., Shekari, F., Mohammadi, M.H., Juhos, K., Végvári, G., Biró, B., 2019. Salt-tolerant plant growth-promoting bacteria enhanced salinity tolerance of salt-tolerant alfalfa (*Medicago sativa* L.) cultivars at high salinity. Acta Physiol. Plant 41 (12), 195.

Ashraf, M., Ali, Q., 2008. Relative membrane permeability and activities of some antioxidant enzymes as the key determinants of salt tolerance in canola (*Brassica napus* L.). Environ. Exp. Bot. 63 (1-3), 266–273.

Ashraf, M.F.M.R., Foolad, M., 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ. Exp. Bot. 59 (2), 206–216.

Bates, L.S., Waldern, R.P., Tear, I.D., 1973. Rapid determination of free proline for water stress studies. Plant Soil 39, 205–207.

Ben Ahmed, C., Magdich, S., Ben Rouina, B., Sensoy, S., Boukhris, M., Ben Abdullah, F., 2011. Exogenous proline effects on water relations and ions contents in leaves and roots of young olive. Amino Acid 40, 565–573.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chakravarty, D., Erande, M.B., Late, D.J., 2015. Graphene quantum dots as enhanced plant growth regulators: effects on coriander and garlic plants. J. Sci. Food Agric. 95 (13), 2772–2778.

Chen, J., Yang, L., Li, S., Ding, W., 2018. Various physiological response to graphene oxide and amine-functionalized graphene oxide in wheat (*Triticum aestivum*). Molecules 23 (5), 1104.

De Freitas, P.A.F., de Carvalho, H.H., Costa, J.H., de Souza Miranda, R., da Cruz Saraiva, K.D., de Oliveira, F.D.B., Coelho, D.G., Prisco, J.T., Gomes-Filho, E., 2019. Salt acclimation in sorghum plants by exogenous proline: physiological and biochemical changes and regulation of proline metabolism. Plant Cell Rep. 38 (3), 403–416.

de Freitas, P.A.F., de Souza Miranda, R., Marques, E.C., Prisco, J.T., Gomes-Filho, E., 2018. Salt tolerance induced by exogenous proline in maize is related to low oxidative damage and favorable ionic homeostasis. J. Plant Growth Regul. 37, 911–924.

Dong, Y.J., Jinc, S.S., Liu, S., Xu, L.L., Kong, J., 2014. Effects of exogenous nitric oxide on growth of cotton seedlings under NaCl stress. J. Soil Sci. Plant Nutr. 14 (1), 1–13.

Eftekhari-Sis, B., Rezazadeh, Z., Akbari, A., Amini, M., 2018. 8-Hydroxyquinoline functionalized graphene oxide: an efficient fluorescent nanosensor for Zn^{2+} in aqueous media. J. Fluoresc. 28 (5), 1173–1180.

El Moukhtari, A., Cabassa-Hourton, C., Farissi, M., Savouré, A., 2020. How does proline treatment promote salt stress tolerance during crop plant development? Front. Plant Sci. 11, 1127.

Faizan, M., Faraz, A., Hayat, S., 2019. Effective use of zinc oxide nanoparticles through root dipping on the performance of growth, quality, photosynthesis and antioxidant system in tomato. J. Plant Biochem. Biotechnol. 29, 553–567.

Faizan, M., Hayat, S., 2019. Effect of foliar spray of ZnO-NPs on the physiological parameters and antioxidant systems of *Lycopersicon esculentum*. Pol. J. Nat. Sci. 34 (1), 87–105.

Food and Agriculture Organization of the United Nations Statistics. FAOSTAT, 2019. Available at: http://faostat3.fao.org.

Fatehi, S.F., Oraei, M., Gohari, G., Akbari, A., Faramarzi, A., 2022. Proline-functionalized graphene oxide nanoparticles (GO–Pro NPs) mitigate salt-induced adverse effects on morpho-physiological traits and essential oils constituents in Moldavian Balm (*Dracocephalum moldavica* L.). J. Plant Growth Regul. 41, 2818–2832.

Ganjavi, A.S., Oraei, M., Gohari, G., Akbari, A., Faramarzi, A., 2021. Glycine betaine functionalized graphene oxide as a new engineering nanoparticle lessens salt stress impacts in sweet basil (*Ocimum basilicum* L.). Plant Physiol. Biochem. 162, 14–26.
Gao, M., Chang, X., Yang, Y., Song, Z., 2020. Foliar graphene oxide treatment increases photosynthetic capacity and reduces oxidative stress in cadmium-stressed lettuce. Plant Physiol. Biochem. 154, 287–294.
Giannopolitis, C.N., Ries, S.K., 1977. Superoxide dismutases occurrence in higher plants. Plant Physiol. 59, 309–314.
Glomstad, B., Altin, D., Sorensen, L., Liu, J.F., Jenssen, B.M., Booth, A.M., 2016. Challenges in characterizing the environmental fate and effects of carbon nanotubes and inorganic nanomaterials in aquatic systems. Environ. Sci. Technol. 50, 2660–2668.
Gohari, G., Panahirad, S., Sadeghi, M., Akbari, A., Zareei, E., Zahedi, S.M., Bahrami, M. K., Fotopoulos, V., 2021a. Putrescine-functionalized carbon quantum dot (put-CQD) nanoparticles effectively prime grapevine (*Vitis vinifera* cv.'Sultana') against salt stress. BMC Plant Biol. 21, 120.
Gohari, G., Zareei, E., Rostami, H., Panahirad, S., Kulak, M., Farhadi, H., Amini, M., Martinez-Ballesta, M.C., Fotopoulos, V., 2021b. Protective effects of cerium oxide nanoparticles in grapevine (*Vitis vinifera* L.) cv. Flame Seedless under salt stress conditions. Ecotoxicol. Environ. Saf. 220, 112402.
Hniličková, H., Hnilička, F., Orsák, M., Hejnák, V., 2019. Effect of salt stress on growth, electrolyte leakage, Na+ and K+ content in selected plant species. Plant Soil Environ. 65 (2), 90–96.
Hummers, W.S., Offeman, RE., 1958. Preparation of graphitic oxide. J. Am. Chem. Soc. 80, 1339.
Khodakovskaya, M.V., De Silva, K., Biris, A.S., Dervishi, E., Villagarcia, H., 2012. Carbon nanotubes induce growth enhancement of tobacco cells. ACS Nano 6 (3), 2128–2135.
Lichtenthaler, H.K., 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods in enzymology. Academic Press, pp. 350–382.
Loreto, F., Velikova, V., 2001. Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. Plant Physiol. 127 (4), 1781–1787.
Lutts, S., Kinet, J.M., Bouharmont, J., 1996. NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. Ann. Bot. 78 (3), 389–398.
Lyon, D.Y., Alvarez, P.J.J., 2008. Fullerene water suspension (nC60) exerts antibacterial effects via ROS-independent protein oxidation. Environ. Sci. Technol. 42, 8127–8132.
Mahmoud, A.W.M., Abdeldaym, E.A., Abdelaziz, S.M., El-Sawy, M.B., Mottaleb, S.A., 2020. Synergetic effects of Zinc, Boron, Silicon, and Zeolite Nanoparticles on confer tolerance in potato plants subjected to salinity. Agronomy 10 (1), 19.
Mohammadi, M.H.Z., Panahirad, S., Navai, A., Bahrami, M.K., Kulak, M., Gohari, G., 2021. Cerium oxide nanoparticles (CeO2-NPs) improve growth parameters and antioxidant defense system in Moldavian Balm (*Dracocephalum moldavica* L.) under salinity stress. Plant Stress 1, 100006.
Murkute, A.A., Satyawati, S.H., Singh, S.K., 2010. Biochemical alterations in foliar tissues of citrus genotypes screened in vitro for salinity tolerance. J. Plant Biochem. Biotechnol. 19, 203–208.
Nakano, Y., Asada, K., 1987. Purification of ascorbate peroxidase in spinach chloroplasts; its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. Plant Cell Physiol. 28 (1), 131–140.
Nakhaie, A., Habibi, G., Vaziri, A., 2022. Exogenous proline enhances salt tolerance in acclimated *Aloe vera* by modulating photosystem II efficiency and antioxidant defense. S. Afr. J. Bot. 147, 1171–1180.
Nasser, M., Cheikh-Ali, H., Hijazi, A., Merah, O., Al-Rekaby, A.E., Awada, R., 2020. Phytochemical profile, antioxidant and antitumor activities of green grape juice. Processes 8 (5), 507.
Park, S., Soon Choi, K., Kim, S., Gwon, Y., Kim, J., 2020. Graphene oxide-assisted promotion of plant growth and stability. Nanomaterials 10, 758.
Pérez-Labrada, F., López-Vargas, E.R., Ortega-Ortiz, H., Cadenas-Pliego, G., Benavides-Mendoza, A., Juárez-Maldonado, A., 2019. Responses of tomato plants under saline stress to foliar application of copper nanoparticles. Plants 8 (6), 151.
Qin, S., Liu, H., Nie, Z., Gao, W., Li, C., Lin, Y., Zhao, P., 2018a. AsA–GSH Cycle and antioxidant enzymes play important roles in Cd tolerance of wheat. Bull. Environ. Contam. Toxicol. 101 (5), 684–690.
Qin, X., Nie, Z., Liu, H., Zhao, P., Qin, S., Shi, Z., 2018b. Influence of selenium on root morphology and photosynthetic characteristics of winter wheat under cadmium stress. Environ. Exp. Bot. 150, 232–239.
Saddiq, M.S., Iqbal, S., Hafeez, M.B., Ibrahim, A.M.H., Raza, A., Fatima, E.M., Baloch, H., Jahanzaib, Woodrow, P., Ciarmiello, L.F., 2021. Effect of salinity stress on physiological changes in winter and spring wheat. Agronomy 11, 1193.
Safikhan, S., Chaichi, M.R., Khoshbakht, K., Amini, A., Motesharezadeh, B., 2018. Application of nanomaterial graphene oxide on biochemical traits of Milk thistle (*Silybum marianum* L.) under salinity stress. AJCS 12 (06), 931–936.
Sairam, R.K., Rao, K.V., Srivastava, G.C., 2002. Differential response of wheat genotypes to long term salinity stress in relation to oxidative stress, antioxidant activity and osmolyte concentration. Plant Sci. 163 (5), 1037–1046.
Schneider, J.R., Caverzan, A., Chavarria, G., 2019. Water deficit stress, ROS involvement, and plant performance. Arch. Agron. Soil Sci. 65, 1160–1181.
Shafiq, S., Akram, N.A., Ashraf, M., Arshad, A., 2014. Synergistic effects of drought and ascorbic acid on growth, mineral nutrients and oxidative defense system in canola (*Brassica napus* L.) plants. Acta Physiol. Plant 36 (6), 1539–1553.
Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. J. Bot., 217037
Siddiqui, Z.A., Parveen, A., Ahmad, L., Hashem, A., 2019. Effects of graphene oxide and zinc oxide nanoparticles on growth, chlorophyll, carotenoids, proline contents and diseases of carrot. Sci. Hortic. 249, 374–382.
Singh, A., Tiwari, S., Pandey, J., Lata, C., Singh, I.K., 2021. Role of nanoparticles in crop improvement and abiotic stress management. J. Biotech. 337, 57–70.
Stefanov, M., Yotsova, E., Gesheva, E., Dimitrova, V., Markovska, Y., Doncheva, S., Apostolova, E.L., 2021. Role of flavonoids and proline in the protection of photosynthetic apparatus in Paulownia under salt stress. S. Afr. J. Bot. 139, 246–253.
Wang, Q., Zhao, S., Zhao, Y., Rui, Q., Wang, D., 2014. Toxicity and translocation of graphene oxide in Arabidopsis plants under stress conditions. RSC Adv. 4 (105), 60891–60901.
Wani, A.S., Ahmad, A., Hayat, S., Tahir, I., 2016. Is foliar spray of proline sufficient for mitigation of salt stress in *Brassica juncea* cultivars? Environ. Sci. Pollut. Res. 23, 13413–13423.
Wani, A.S., Ahmad, A., Hayat, S., Tahir, I., 2019. Epibrassinolide and proline alleviate the photosynthetic and yield inhibition under salt stress by acting on antioxidant system in mustard. Plant Physiol. Biochem. 135, 385–394.
Wani, K.I., Naeem, M., Castroverde, C.D.M., Kalaji, H.M., Albaqami, M., Aftab, T., 2021. Molecular mechanisms of nitric oxide (NO) signaling and reactive oxygen species (ROS) homeostasis during abiotic stresses in plants. Int. J. Mol. Sci. 22 (17), 9656.
Yang, J., Cao, W., Rui, Y., 2017. Interactions between nanoparticles and plants: phytotoxicity and defense mechanisms. J. Plant Interact. 12, 158–169.
Zhao, D., Fang, Z., Tang, Y., Tao, J., 2020. Graphene oxide as an effective soil water retention agent can confer drought stress tolerance to *paeonia ostii* without toxicity. Environ. Sci. Technol. 54, 8269–8279.