

Responses of selected C3 and C4 halophytes to elevated CO₂ concentration under salinity

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Halophytes have superior capacity to withstand soil salinity and are appropriate resources to study the mechanism of salt tolerance which can be harnessed to develop crops to withstand salinity. In this communication, we report the effect of salinity (200 mM NaCl) and elevated carbon dioxide (CO₂) treatments in tandem, on select halophytes that have different photosynthetic pathways: C3 and C4. The plants were raised in ambient (380 ppm) and enriched (500 ppm) concentrations of CO₂ using a mini-FACE facility. Total chlorophyll content, total soluble sugar concentration, lipid peroxidation level and electrolyte leakage were measured from fresh leaf samples collected at different time points. The results show a positive effect for elevated CO₂ concentration on salt tolerance in both C3 and C4 plants, and indicate that halophytes may benefit from rising atmospheric CO₂ concentration. The results also suggest that C4 halophytes may benefit from the rising atmospheric CO₂ concentration than C3 halophytes.

Keywords: Elevated carbon dioxide, halophytes, salinity, photosynthetic pathway, *Sesuvium portulacastrum*, *Suaeda nudiflora*.

ALTHOUGH the earth's climate has never been static, the planet is currently experiencing faster changes in climate/weather patterns than it had in the past, due to increased anthropogenic activities. Since the beginning of the Industrial Revolution, carbon dioxide (CO₂) concentration in the atmosphere has increased from 280 to approximately 400 ppm (ref. 1) and is estimated to increase by 2.25 ppm each year according to the US National Oceanic and Atmospheric Administration (NOAA). Increasing concentrations of greenhouse gases (GHGs), including CO₂ are directly linked to changes in temperature, rainfall, near-surface radiation, higher solar radiation and desertification²⁻⁴. Soil salinization affects almost 7% of the total global land area and 20–50% of

the global irrigated farmland, and is largely due to desertification⁵.

Soil salinity is a major abiotic stress that impacts plant growth and productivity⁶. High salt concentrations decrease water uptake leading to water stress and inhibit key metabolic processes such as photosynthesis. Soil salinization also inhibits plant growth due to osmotic and ionic stress⁷. High soil salinity leads to enhanced production of reactive oxygen species (ROS) in plants, which is accompanied by increased membrane lipid peroxidation⁸. Most plant species are sensitive to salinity even at low concentrations (glycophytes), while some species can tolerate and complete their life cycle even at high saline concentrations (halophytes).

Increase in atmospheric CO₂ coupled with salinity adds another component to the range of complex physiological and morphological responses in plants that directly impact photosynthetic processes. Plant response to increased CO₂ concentration and/or salinity differs, and is based on the photosynthetic pathways followed, which impacts growth rates and other factors. Most species that use the C3, photosynthetic pathway respond favourably to elevated atmospheric CO₂ (refs 9, 10). However, long-term exposure to elevated levels of CO₂ has been reported to substantially suppress photosynthesis¹¹. Many plant species that use C4 and CAM pathways respond positively to increase in atmospheric CO₂ too, but the responses are generally less vigorous than that of C3 plants^{12,13}.

At increased atmospheric CO₂ concentration, plants perhaps counteract the water stress better, caused due to salinity, by reducing transpiration and increasing water potential¹⁴. As more carbon dioxide is assimilated due to greater diffusion of this gas into the leaf, it causes an increase in the supply of carbohydrates. Increased carbohydrate concentration will reduce the osmotic potential and hence turgor pressure is maintained¹⁵. Additionally, plants use water more efficiently due to decrease in stomatal conductance^{16,17}. Some studies support the hypothesis that CO₂ enrichment stimulates plant growth via increased photosynthesis and not necessarily improved water relations¹⁸. All these reports point towards the need for more comprehensive studies on the interactions between elevated CO₂ and salinity conditions with different plant species.

Hence in this study, we evaluated the combined effect of salinity and elevated CO₂ on photosynthetic pigment content, lipid peroxidation level, electrolytic leakage and osmolyte concentration (total soluble sugars) of two halophytes. *Sesuvium portulacastrum* (Aizoaceae) is an important mangrove-associated, facultative halophyte and follows the C3 pathway. This plant has the ability to grow under high salinity with lower nutrition availability; it is used as a soil cover and landscaping plant, and plays a vital role in alleviating saline soil, desalination, desert greenification and as an alternative to problem soils in

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arid and semiarid conditions¹⁹. *Suaeda nudiflora* (Chenopodiaceae) is a halophyte that grows in the wild, in the high saline and extreme high tidal belt. As the seeds contain approximately 30–35% oil, this plant has a potential to be a future oil crop and is also suitable for producing high protein biomass in saline soils as it is a C4 plant²⁰. Our research findings record plant metabolic responses to combined salt and elevated CO₂ levels. The changes in metabolic and physiological responses between plants following the C3 and C4 pathways are presented in this communication.

Seeds of *S. portulacastrum* were collected from Tamil Nadu and *S. nudiflora* from Andhra Pradesh, India. The seedlings were germinated in vermiculite-filled pots kept in plastic trays, in modified Hoagland's medium²¹ which was changed every week, under greenhouse conditions with average temperature of 30°C/22°C (day/night). The CO₂ enrichment experiments were conducted at M.S. Swaminathan Research Foundation, Chennai, using the mini-free air CO₂ enrichment (FACE) facility on *S. portulacastrum* and *S. nudiflora*. The plants were cultured hydroponically in Hoagland's solution, pH 5.7 (ref. 22). The medium was replenished every 14 days and up to 5 litres of medium was poured daily in each tank to maintain the water level lost by evapo-transpiration. After a month, plants from two of the four tanks were acclimatized to 500 ppm of CO₂ during daylight hours for one month and labelled as CO₂ rings. Plants in the remaining two tanks continued to grow under atmospheric air and were labelled as ambient rings. After acclimatization, 200 mM NaCl was added to one tank each from ambient and CO₂ rings with continued exposure to the gas in the CO₂ ring. Thus, the treatment conditions imposed were ambient control (AC), ambient NaCl (ACS), CO₂ (EC) control and CO₂ NaCl (ECS). The treatments were given for three months. The leaf samples from each treatment were collected at five time points, i.e. on 30th, 45th, 60th, 75th and 90th day.

Lipid peroxidation was determined by measuring malondialdehyde (MDA) content as described by Hodges *et al.*²³. Fresh leaves were homogenized in 5% trichloroacetic acid (TCA) solution. To 2 ml of extract, 3 ml of 0.5% thiobarbituric acid (TBA) and 5% trichloroacetic acid (TCA) were added, and then heated at 90°C for 30 min and cooled in ice to room temperature. Samples were centrifuged at 5000 rpm for 15 min. The supernatant was assayed for absorbance at 450, 532 and 600 nm.

Total soluble sugars were determined following the method of Yadav *et al.*²⁴. Fresh leaves were ground in 95% ethanol and centrifuged at 3500 g for 10 min. To 100 µl of supernatant 3 ml of freshly prepared anthrone reagent was added (150 mg of anthrone in 100 ml of 72% H₂SO₄). The mixture was placed in a boiling water bath for 10 min. The reaction was stopped by chilling the tubes in ice. Absorbance was estimated at 625 nm.

The electrical conductivity was determined as described in Lutts *et al.*²⁵. Leaf discs of similar size were washed thoroughly with deionized water. The discs were kept in closed vials containing 10 ml of deionized water and incubated at 25°C on a rotary shaker for 24 h. The electrical conductivity (L_t) was determined using conductivity meter. Samples were autoclaved (120°C for 20 min) and cooled up to 25°C, and electrical conductivity (L_0) was again measured.

The chlorophyll content was determined following the method of Arnon²⁶. Leaves (0.5 g) were homogenized in 80% acetone and incubated for 1–2 h with shaking at 120 rpm in dark. The homogenate was centrifuged at 3000 rpm for 5 min in dark and absorbance of the supernatant was measured at 645 nm and 663 nm.

Each experiment was carried out in three biological triplicates. All data obtained were expressed as mean, SD and subjected to analysis of variance (ANOVA) to determine the significance of difference between the means of control and treated plants for every set of treatments. A Tukey HSD multiple comparison of mean test was used, the means were separated with the least significance difference (LSD) test at a confidence level of 0.05.

Chlorophyll content in *S. portulacastrum* salt-treated leaf tissue (SpACS) was less than control leaves (SpAC) at 30 days interval, but as the treatment progressed the treated samples showed more chlorophyll content than control samples. *S. portulacastrum* plants treated with elevated CO₂ (SpEC) had less chlorophyll content than that of control plants (SpAC) on days 30, 45 and 60 of treatment. However, subsequently they showed an increase in chlorophyll content with 31.81% and 41.57% increase on day 75 and day 90 samples respectively, compared to SpAC samples. Under salt and elevated CO₂ treatment, chlorophyll content of *S. portulacastrum* plants (SpECS) showed increase at all time points except day 60, compared to SpAC, SpACS and SpEC plants (Figure 1).

Chlorophyll content in *S. nudiflora* plants treated with salt (SnACS) showed slight increase at days 30 and 45 compared to control plants (SnAC), but at later time points of treatments it showed a sharp decrease. Under treatment of elevated CO₂, *S. nudiflora* samples (SnEC) showed an increase in chlorophyll content compared to control samples (SnAC) at all time points analysed, with 140.19% increase in chlorophyll content at day 90 of treatment. Under combined treatment of salt and elevated CO₂, *S. nudiflora* plants (SnECS) showed less chlorophyll content than SnEC samples at days 30, 45 and 60, but showed increase on days 75 and 90. Similarly, chlorophyll content of SnECS samples was greater than SnAC and SnACS samples at later stages of treatments. At days 75 and 90 of treatment, SnECS, samples showed 282.75% and 364.66% increase in chlorophyll content respectively, compared to SnACS samples (Figure 1).

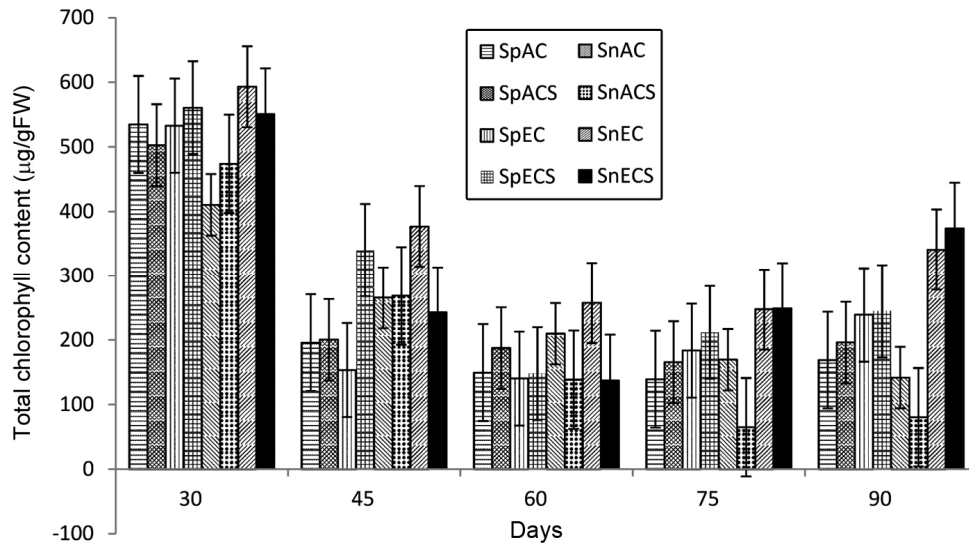


Figure 1. Effect of salt treatment and/or elevated CO₂ on total chlorophyll content of *Suaeda nudiflora*. Vertical bars indicate standard errors of the mean for each treatment. Values indicate significant differences between treatments based on LSD values ($P < 0.05$).

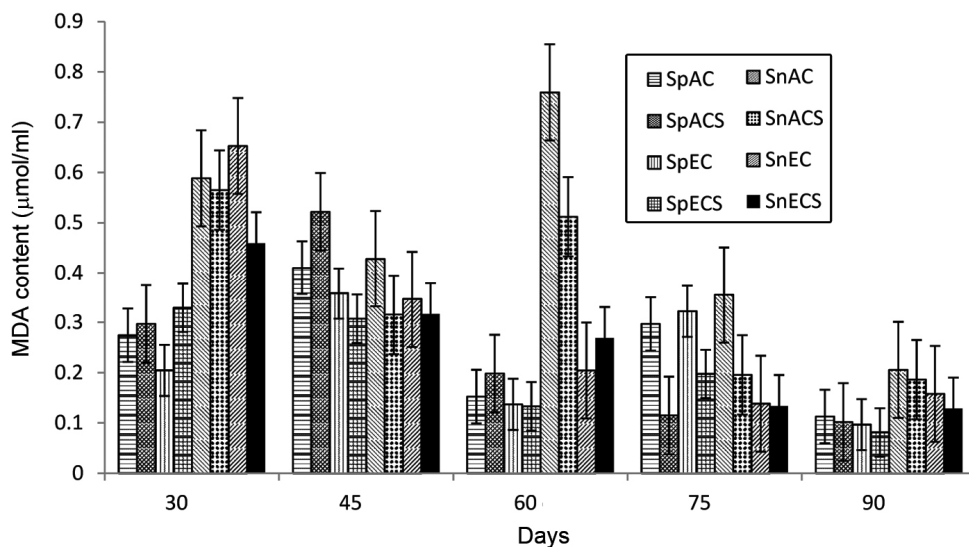


Figure 2. Effect of salt treatment and/or elevated CO₂ on malondialdehyde (MDA) content of *S. nudiflora*. Vertical bars indicate standard errors of the mean for each treatment. Values indicate significant differences between treatments based on LSD values ($P < 0.05$).

Lipid peroxidation analysis of *S. portulacastrum* salt-treated leaf tissue (SpACS) showed an increase in MDA content on days 30, 45 and 60 of salt-treatment, and decrease on days 75 and 90 compared to SpAC samples. Under the treatment of elevated CO₂, MDA content in SpEC plants was less than SpAC plants in all time points, except for a slight increase (8.41%) at day 75. *S. portulacastrum* leaf tissue under the combined treatment of elevated CO₂ and salt (SpECS) showed less MDA content at days 45, 60 and 90 compared to SpACS and SpAC samples (Figure 2).

In *S. nudiflora* plants, after salt treatment (SnACS) MDA content was less than that in control plants (SnAC)

at all time points. Similarly, MDA content after elevated CO₂ treatment (SnEC) was higher than control (SnAC) at all stages of treatment, except on day 30. Plants under elevated CO₂ and salt treatment (SnECS) had reduced MDA content than those under salt treatment (SnACS) at all time intervals, except day 45, where it showed a mild increase in MDA content. SnECS samples had less MDA than SnAC samples at all time points, and the percentage of decrease was higher than that in *S. portulacastrum* at corresponding time points (Figure 2).

Electrical conductivity measurements on *S. portulacastrum* showed that samples treated with salt (SpACS) showed a higher value at all time points compared to

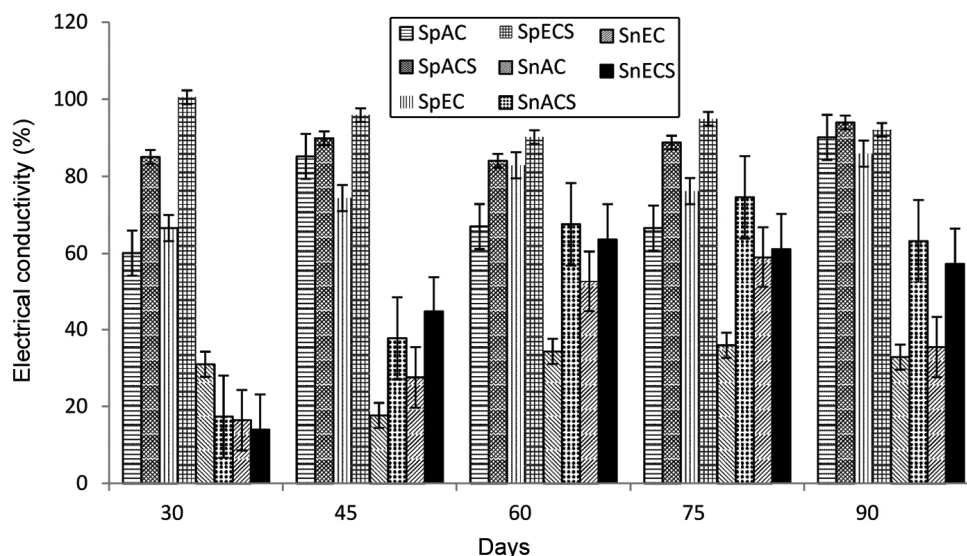


Figure 3. Effect of salt treatment and/or elevated CO₂ on electrical conductivity of *S. nudiflora*. Vertical bars indicate standard errors of the mean for each treatment. Values indicate significant differences between treatments based on LSD values ($P < 0.05$).

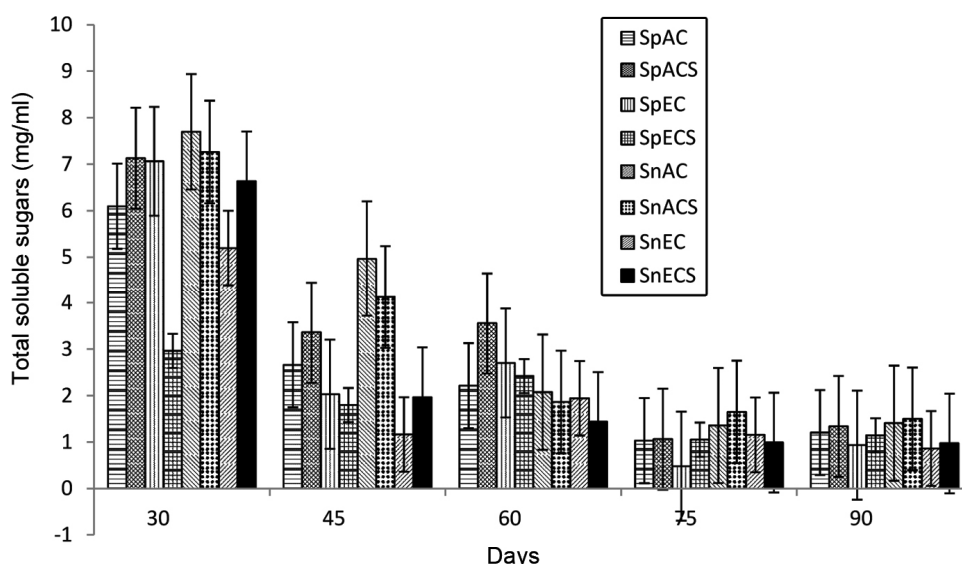


Figure 4. Effect of salt treatment and/or elevated CO₂ on total soluble sugars of *S. nudiflora*. Vertical bars indicate standard errors of the mean for each treatment. Values indicate significant differences between treatments based on LSD values ($P < 0.05$).

control (SpAC), but the difference was less marked on day 90. Elevated CO₂ treatment (SpEC) showed increase in electrical conductivity at three time points and decrease at two time points compared to SpAC. Combined treatment of elevated CO₂ and salt (SpECS) resulted in increased electrical conductivity at all time points compared to SpAC, but the percentage of increase was less than that shown by salt alone (SpACS) at later time points (Figure 3).

S. nudiflora samples under salinity (SnACS) showed increase in electrical conductivity compared to control

(SnAC) at all time points, except on day 30. Elevated CO₂ treatment (SnEC), however, increased electrical conductivity at three time points and decreased it at two, compared to control (SnAC). Combined treatment of salt and elevated CO₂ (SnECS) reduced the electrical conductivity at all time points, except day 45, compared to salt treated samples (SnACS; Figure 3).

Quantification of total soluble sugars in *S. portulacastrum* under salinity (SpACS) showed a higher value compared to control (SpAC) samples at all time points, although the difference was less marked on days 75 and

90 of treatment. Under elevated CO₂ treatment (SpEC), however, total soluble sugars were found to be less than in SpAC samples at three time points. Total soluble sugars after treatment with elevated CO₂ and salt (SpECS) was found to be less than SpACS and SpAC at all time points (Figure 4).

Measurement of total soluble sugars in *S. nudiflora* after salt treatment (SnACS) decreased at initial time points and increased at later time points compared to control (SnAC) samples. Treatment with elevated CO₂ (SnEC) decreased total soluble sugars at all time points compared to SnAC. Elevated CO₂ treatment along with salt treatment (SnECS) reduced total soluble sugars at all time points compared to SnACS and SnAC (Figure 4).

Abiotic stresses, namely drought, salinity, extreme temperature, etc. are known to reduce the contents of photosynthetic pigments. The initial decrease in chlorophyll content in *S. portulacastrum* after salt treatment followed by increase at later time points indicate that the plants are probably acclimatizing to the stress after the initial shock. The observations also show a positive effect of elevated CO₂ concentration on chlorophyll content as the length of treatment increased. Increased CO₂ concentration during salt treatment had a positive effect on chlorophyll content and indicates that the plants might survive salt treatment better under these conditions. Contrary to the results for *S. portulacastrum*, *S. nudiflora* plants showed higher chlorophyll content at initial stages of treatment indicating better initial stress tolerance. However, the sharp decrease in chlorophyll content at later stages indicates that prolonged salt stress is perhaps more deleterious to *S. nudiflora*. The percentage increase in chlorophyll content in *S. nudiflora* was much higher than *S. portulacastrum* at corresponding time points under the treatment of elevated CO₂, indicating a more positive effect on chlorophyll content in *S. nudiflora* than *S. portulacastrum*. This is in accordance with the report on studies on maize (C₄ plant) and soybean (C₃ plant) at elevated CO₂ treatment²⁷. Salinity reduced the positive effect of chlorophyll content with elevated CO₂ treatment in the initial stages of stress. However, at later stages of stress, elevated CO₂ treatment was shown to have a high positive effect on chlorophyll content in *S. nudiflora* plants under salinity.

It is well documented that increased levels of toxic cation, Na⁺ under salt stress leads to breakdown of chlorophyll (Chl)²⁸ and reduction in photosynthetic pigments, such as Chl *a* and *b* under abiotic stresses has been reported in many crops^{29–32}. The salt-induced alterations in leaf chlorophyll content could be due to impaired chlorophyll biosynthesis or accelerated pigment degradation. Researchers have shown decrease in chlorophyll precursors such as glutamate and 5-aminolaevulinic acid (ALA) under salt stress, indicating that salinity affects chlorophyll biosynthesis more than chlorophyll breakdown^{29,33–35}.

Although salt stress reduces the chlorophyll content, the extent varies based on salt-tolerance of the plant species. Chlorophyll content was shown to increase in salt-tolerant species and decrease in salt-sensitive species under salinity^{29,36,37}. Hence, accumulation of chlorophyll serves as a useful biochemical indicator of salt tolerance in crops^{29,38–40}.

Plants respond to environmental stresses through generation of ROS which cause oxidative damage to many cellular components, including membrane lipids. Lipid peroxidation levels, measured as MDA content, have been considered as an indicator of salt-induced oxidation in cell membranes and a tool for determining salt tolerance in plants^{41–43}.

Salinity initially increased the level of lipid peroxidation in *S. portulacastrum*. However, at later stages of treatment, a decrease was observed, possibly due to the plants acclimatizing to the stress. Treatment with elevated CO₂ reduced the level of lipid peroxidation in the plants compared to the untreated plants, indicating that elevated CO₂ could be beneficial to the species when the plants are not under stress. Elevated CO₂ was found to have a positive effect on limiting salt-induced lipid peroxidation at later stages of stress. Similar to *S. portulacastrum* results, elevated CO₂ showed a positive effect on limiting salt-induced lipid peroxidation at all stages of stress treatments in *S. nudiflora*.

Soluble sugars play a major role in the synthesis of other compounds, production of energy, stabilization of membranes, etc. and act as signal molecules and regulators of gene expression^{44,45}. Carbohydrates help in the maintenance of osmotic balance, osmoprotection and ROS scavenging, and in carbon storage during stress⁴⁶. Soluble sugars have been shown to be responsible for up to 50% of the total osmotic potential in glycophytes subjected to saline stress^{47–52}. Hence soluble sugar content is a good indicator of abiotic stress tolerance in plants. In durum wheat, for example, soluble sugar content was a better marker for selecting drought-tolerant cultivars than proline content⁵³.

Our results showed that under salinity, higher amount of total soluble sugars was present in *S. portulacastrum*. However, elevated CO₂ treatment did not show increase in total soluble sugars, and total soluble sugars in samples treated with elevated CO₂ and salt were found to be less than that in control samples. Similar results were observed in *S. nudiflora*. This corroborates with an earlier report analysing the growth and carbohydrate metabolism in three rice cultivars differing in salinity stress tolerance, which reported increased concentration of sugars in response to salinity stress only in sensitive cultivar and not in tolerant cultivars^{54,55}. Another report on tomato showed that salt-sensitive cultivar was able to accumulate hexoses and sucrose under salinity stress treatments while in salt-tolerant cultivar, their concentrations remained unchanged or decreased⁵⁶. A decrease in total soluble sugars

in this study is probably due to salt-tolerant nature of the selected plants.

Electrolyte leakage is widely used as a measure for stress-induced injury of plant tissues and as an indicator of plant stress tolerance. The electrolyte leakage is ubiquitous among different species, tissues and cell types, and can be triggered by all major stress factors, including pathogen attack, salinity, heavy metals, oxidative stress, high soil acidity (pH < 4), wounding, waterlogging, drought, heat and others⁵⁷. Reduced electrical conductivity of salt-treated samples at elevated CO₂ showed a positive effect for elevated CO₂ treatment in salt tolerance of *S. nudiflora* and *S. portulacastrum*.

Elevated CO₂ concentration is effective in offsetting physiological damages due to salinity stress in the plants studied, indicating that halophytic plants may gain from rising atmospheric CO₂ concentration in the scenario of climate change. This study shows significant differences in responses of plants following C3 and C4 pathway, to salt and/or elevated CO₂ treatments. *S. nudiflora* (C4) was more sensitive to 200 mM NaCl than *S. portulacastrum* (C3), as indicated by significant decrease in total chlorophyll content and significant increase in MDA content. Elevated carbon dioxide ameliorates the effects of salt in the selected plants. This positive effect of elevated CO₂ was more significant in C4 (*S. nudiflora*) plant on extended exposure time than in plants following the C3 pathway, where the positive effects of elevated CO₂ in salt treatment were more gradual. Both plants showed negative and varying response at the 30 day (initial time point) time point, to salt under elevated CO₂ which turned to positive and uniform at longer, extended time points. This suggests that 500 ppm of CO₂ takes a longer time to show a positive effect on these select halophytes differing in their photosynthetic pathways. In the context of accelerated climate change, these results indicate that prolonged atmospheric CO₂ enrichment may provide greater protection to the salt-sensitive C4 halophytes than the less-sensitive C3 halophytes. These results pave the way for a better understanding of plant responses to salinity under increased atmospheric CO₂ concentration.

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