Differential Accumulation of Metabolites in *Suaeda* Species Provides New Insights into Abiotic Stress Tolerance in C₄-Halophytic Species in Elevated CO₂ Conditions

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Abstract: Halophytic plants can adapt to grow and thrive in highly saline conditions. *Suaeda* species are annual halophytes with high salt tolerance and are most suitable in the restoration of salinized or contaminated saline land and as food, forage, medicine, and bioenergy. In this study, we comprehensively analyzed the different metabolic responses of *Suaeda* species under salt and drought stress at ambient and elevated CO₂ conditions. Seedlings of *Suaeda* species were treated with 500 mM NaCl and 5% of polyethylene glycol under elevated CO₂ stress conditions for 24 h. Then, widely untargeted metabolites were detected by gas chromatography–mass spectrometry. Different metabolites involved in amino acid metabolism, glycolysis, photorespiration, and tricarboxylic acid cycle were quantitatively determined after stress treatments. A total of 61 primary metabolites were annotated. Different treatments increased the contents of certain metabolites, such as amino acids, sugars, and organic acids, as well as some antioxidants, such as quininic acid, kaempferol, and melatonin. These substances may be correlated with osmotic tolerance, increased antioxidant activity, and medical and nutritional value in the species. This study suggests that various metabolites differentially accumulated in C₄ *Suaeda* species under varying stress conditions. Furthermore, this work provides new insights into the key secondary metabolite pathway involved in stress tolerance.

Keywords: abiatic stress; C₄ plants; drought; elevated CO₂; halophyte; salinity

1. Introduction

Plants are affected continuously by various environments, such as abiatic and biotic factors, although the consequences of these factors on the plant system depends on their intensity or quantity. Abiotic stresses, including elevated CO₂, high temperature, lower precipitation, cold, salinity, drought, heavy metals, and different oxidative stress, are the primary sources of the comprehensive loss of agricultural productivity, total biomass yield, and crop quality [1–3]. Elevated CO₂ increases the net photosynthesis and water use efficiency in C₃ and C₄ plants, and this may also compensate for savings in growth and plant yield under drought stress [4–7]. C₃ plants were previously reported to increase their net photosynthesis rate and growth by about 35% under enhanced CO₂ conditions, stimulating biomass production [8,9]. Elevated CO₂ can also diminish the effects of abiatic stresses on plants, including heat, ozone, and drought [10]. Particularly, the mitigation
of drought stress responses is triggered by stomatal factors, including increased stomatal closure and reduced stomatal density, improving the water use efficiency in plants [11,12].

Drought and salinity stresses are severe threats to plant growth and photosynthesis metabolism due to recurring global climate changes. Photosynthesis inhibition, alterations of cell metabolism, and deterioration of proteins and membranes are commonly detected under stress conditions. Worldwide, salinization is a significant problem. Approximately 2% of total land is affected by salinity, and 45 million ha of the available 230 million ha of irrigated land is salt-affected [13]. To cope with these environmental changes, plants induce numerous physiological, biochemical, and molecular changes by activating corresponding key genes essential for plant defense mechanisms [14]. Recent studies have revealed that plants respond within seconds to minutes to different stress stimuli through different physiological, biochemical, metabolic, and molecular networks [15]. Some rapid responses include electrolyte leakage, membrane stability, antioxidant enzyme activities, stomatal conductance changes, quenching of reactive oxygen species (ROS), accumulation of osmolytes, and expression of key genes involved in signal pathways and defense systems [15]. Therefore, elevated ROS levels and imbalances in redox homeostasis lead to changes in metabolites, creating oxidative stress [16]. However, understanding the metabolic responses in plant systems under salt and drought effects and under predicted future climate conditions could help identify new strategies to improve stress tolerance.

Halophytes have a remarkable ability to tolerate high salinity and adverse drought stress during their lifespan [17]. Taxa with C_4 carbon fixation appear to be overrepresented among halophytes. They have evolved from C_3 ancestors accordingly to survive in these environmental stress conditions. Further, they (halophytes) develop efficient features, such as a high photosynthesis rate, uptake or elimination of selective ions, synthesis of the enzymatic and non-enzymatic antioxidant defense system, alterations in metabolite levels, and high gene expression compared to glycophytes, in order to cope with induced stress through environmental changes [18,19]. Previous studies have demonstrated that plants accumulate low molecular weight organic compounds, including sugars, proline, and glycine betaine, and act as an osmoprotectant under salt stress in halophytes [20]. Halophytes are most suitable for the restoration of salinized or contaminated saline land for removing heavy metals from saline soils. They can be utilized as food, forage, medicine, and bioenergy [21–24]. Halophytes are a good source of salt-resistant genes [25–36] and promoters [37–40].

Due to favorable climate and edaphic conditions, one of the world’s best salt marsh flora occurs along a 5700 km stretch of coastline in India and Saudi Arabia. The higher plants present in this vegetation are mangroves, halophytes, and sea grasses. An extreme halophyte, *Suaeda*, an annual plant, also known as seep weed or sea blite, belongs to the Amaranthaceae family, is widely dispersed on India’s east and west coasts, and completes its life cycle in saltmarsh areas, such as coastal and intertidal regions near estuaries. Both *Suaeda monoica* and *Suaeda fruticosa* possess unique C_4 carbon assimilation, in which the photosynthesis pathway occurs within a single elongated chlorenchyma cell [41]. The optimal growth range for both *Suaeda* species is between 200 and 500 mM NaCl [42]. To deal with this stressed environment and to conserve water accessibility, halophytes accumulate compatible solutes, such as glycine betaine, sucrose, and proline, in vacuoles together, maintaining osmotic potential via the uptake of osmotically active ions [43]. Naturally, C_4 plants have a higher photosynthetic rate and water use efficiency compared to C_3 plants. Enzymes involved in C_4 carbon assimilation also play a fundamental role in plant defense responses under different abiotic and biotic stress conditions [44,45].

Metabolite profiling is one of the efficient and quantitative methodologies that provide a functional analysis approach in order to connect physiological and metabolic responses to phenotypic and genetic information [46–51]. Such technologies can be used to better understand the mechanisms underpinning plant responses to global climate change [52,53]. A metabolomics study estimating the nutritional value examined whether these metabolites are associated with stress tolerance [54,55]. This technique can be used to provide
perceptions into metabolic pathways and instabilities during stress; therefore, it can reveal targets for improving plant performance in the future. A previous omics study showed that deviations in the nature and amount of metabolites can show how the plant acclimatizes to environmental changes [56]. Metabolites are anxious under stress conditions, and the plant system needs to regulate the metabolite levels to maintain basal metabolism and reach new homeostasis [57]. Therefore, metabolomics is the most direct tool for studying metabolite changes under different stress conditions [58]. During plant stress, changes in primary metabolites are most prominent and also show a general trend of response to abiotic stress. This involves the accumulation of compatible solutes, such as amino acids, sugars, and sugar alcohols, to cope with osmotic stress [59]. Several reports are available on the metabolic responses of C₄ plants, while the roles of different amino acids and metabolites of the tricarboxylic acid cycle (TCA) cycle have been evaluated under different abiotic stress treatments in *Suaeda* species [20,60–64]. Briefly, some specific metabolites and their derivatives, including nucleotides, amino acids, organic acids, lipids, antioxidants such as quercetin, and intermediates of the TCA cycle, increase in *Suaeda salsa* under salt stress, confirming their role in tolerance [65,66].

Similarly, the concentrations of hexose phosphate, intermediates of the TCA cycle, and osmoprotectant metabolites increase concomitantly with salt concentrations in a salt-tolerant variety of *Hordeum vulgare* [67]. Moreover, in *Zea mays*, a significant accumulation of organic solutes plays an essential role in osmotic stress resistance [68]. Furthermore, metabolites play indispensable roles in stress tolerance [57,69], nitrogen metabolism [70,71], regional differences [72], and phenotypic changes [73]. Interestingly, C₄ guinea grasses (*Panicum maximum* Jacq.) alter their transcript and metabolite profiling associated with environmental response, stomatal function, and secondary metabolism under elevated CO₂ and temperature [69]. Overall, previous reports have shown that metabolites play an essential role in providing plant tolerance under different stress conditions.

Previous investigations have proven that *S. monoica* and *S. fruticosa* are two *Suaeda* species that exhibit distinct physiological performance under elevated CO₂ stress [74,75]. There is an increasing need to investigate and understand plant metabolic responses in terms of biochemical and physiological levels to global climate change or abiotic stress. With a broad objective of assessing how elevated CO₂ can mitigate the detrimental effects of plants under stress conditions, this study was carried out to investigate whether these two *Suaeda* species respond differently under salt and drought stress conditions under ambient and elevated CO₂ conditions (in a plant growth chamber) followed by stress treatments with salt (500 mM NaCl) and osmotic 5% PEG for 24 h. The TCA cycle were quantitatively determined after stress treatments. The effects of elevated CO₂ integrated with salt or drought stress on the physiological and metabolic adaptation mechanisms of both *Suaeda* species were investigated for the first time.

2. Materials and Methods
2.1. Plant Materials and Stress Treatments

Seeds of *S. monoica* and *S. fruticosa* were germinated in garden soil and irrigated with a half-strength nutrient solution [74]. Seedlings were allowed to grow in a culture room under control conditions with a 12 h light/12 h dark cycle at 25 °C ± 2 °C. After 2 months, seedlings were initially cultivated in half-strength Hoagland hydroponic culture medium [76] for 1 month. Three-month-old *Suaeda* plants were divided into two sets. The first set of plants was shifted to a plant growth chamber (Percival, Iowa, USA) under ambient CO₂ (~400 ppm) conditions, then acclimatized plants were subjected to salt (500 mM NaCl) and osmotic (5% polyethylene glycol (PEG)) stress for 24 h. The second set of plants was acclimatized in elevated CO₂ (~900 ppm) conditions (in a plant growth chamber) followed by stress treatments with salt (500 mM NaCl) and osmotic 5% PEG for 24 h. All stress treatments were carried out under plant growth chamber conditions involving a 12 h light/12 h dark photoperiod at 25 °C ± 2 °C, 1100 µmol quantum m⁻² s⁻¹ light, and 55% to 60% relative humidity. Mature fresh leaves of all treated plants and the
corresponding control plants were harvested, frozen in liquid N\(_2\), and stored at \(-80^\circ\text{C}\) for further analysis.

2.2. Metabolite Extraction

Metabolites were extracted from leaves of control and treated plants and analyzed by gas chromatography–mass spectrometry (GC-MS) [77]. Leaf samples (~0.1 g) were ground into fine powder in liquid N\(_2\), then ice-cold 100% methanol (0.7 mL) was added for enzyme inactivation, followed by the addition of 30 µL adonitol (0.2 mg/mL) as an internal reference [78]. The samples were mixed by vortexing for 20 s, incubated at 70 °C, sonicated for 10 min at room temperature, then centrifuged at 10,000× g for 10 min. A clear solution was transferred into a 2 mL tube, approximately 325 µL chloroform and 700 µL water were added, then the samples were mixed thoroughly and centrifuged at 10,000× g for 5 min at room temperature. The upper polar phase (600 µL) was transferred in a fresh 1.5 mL tube, dried in a vacuum concentrator (without heating), then stored at \(-80^\circ\text{C}\) for further analysis. Before derivatization, the samples were again vacuum dried for 30 min, then 60 µL methoxyamine hydrochloride (20 mg/mL in pyridine) was added, followed by incubation for 2 h at 37 °C in shaking conditions. Finally, 130 µL N-methyl-N-(trimethylsilyl) trifluoroacetamide was added and incubated for 30 min at 37 °C. The sample was transferred into glass vials for GC-MS analysis.

2.3. Metabolite Analysis by GC-MS

The GC-2010 instrument (Shimadzu, Kyoto, Japan, model GC-MS TQ8040) was used for the analysis in collaboration with King Saud University. The chromatographic parameters were as follows: column, RTX-5MS (diphenyl dimethyl polysiloxane: 30.0 m × 0.25 mm); injection, split injection; injection volume, 1 µL; split ratio: 50.0. The initial temperature was 80 °C with a hold time of 2 min; after this, the temperature was raised to 315 °C with a rate of 10 °C/min and held for 15 min. Helium gas was used as a carrier, with a flow rate of 2 mL/min, and the total processing time was 40 to 50 min. The obtained picks were analyzed by matching with the available NIST GC-MS database and the concentration (calculated using the peak area of the internal reference adonitol) was expressed as µg/g fresh weight.

2.4. Statistical Analysis and Data Visualization

Metabolomics datasets were analyzed by multivariate analysis methods, including principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal projections to latent structures discriminant analysis (OPLS-DA) [79]. The Kyoto Encyclopedia of Genes and Genomes database was used to annotate metabolites and different heatmaps were constructed to represent the differential expression of metabolites under varying stress conditions.

3. Results

3.1. Differential Accumulation of Metabolites under Different Stress Conditions

Metabolites were extracted from both Suaeda plants grown under different abiotic stress conditions and analyzed by GC-MS. A total of 61 primary metabolites were detected in the leaves of both Suaeda species under different conditions (Table 1). These identified metabolites belong to different groups: 14 amino acids, 20 sugars, 11 sugar acids, 6 fatty acids, 9 different compounds, and 1 flavonoid (kaempferol). These metabolites were commonly observed and quantified in various abiotic stress conditions in both Suaeda species. Of the 14 amino acids, some are nutraceutical essential amino acids that are necessary ingredients for functional food and play a vital role in human health, including the proper functioning of numerous biosynthesis mechanisms.
| Metabolites          | Ambiente CO₂ (400 ppm)                  | Elevated CO₂ (900 ppm)                  |
|---------------------|----------------------------------------|----------------------------------------|
|                     | Suáeda monoica                        | Suáeda fruticosa                       |
|                     | Control                                | Salt                                   | Control                                | Salt                                   |
| Amino acids         |                                        |                                        |                                        |                                        |
| Asparagine          | 0.39 ± 0.01                            | 3.67 ± 0.27                            | 2.27 ± 0.19                            | -                                      |
| Citramalic acid     | 2.08 ± 0.18                            | 0.45 ± 0.01                            | 0.46 ± 0.01                            | 0.41 ± 0.01                            |
| Glutamic acid       | 6.56 ± 0.05                            | 38.04 ± 2.86                           | 8.28 ± 0.01                            | 4.86 ± 0.01                            |
| Glutamine           | 0.29 ± 0.01                            | 6.71 ± 0.50                            | 6.16 ± 0.61                            | 0.72 ± 0.01                            |
| Norvaline           | 0.17 ± 0.01                            | -                                      | 0.25 ± 0.01                            | 0.09 ± 0.01                            |
| Proline             | 0.40 ± 0.02                            | 10.80 ± 0.83                           | 6.82 ± 0.60                            | 0.41 ± 0.01                            |
| Serine              | 2.30 ± 0.02                            | 16.86 ± 1.28                           | 11.99 ± 104                            | 1.55 ± 0.01                            |
| Threonine           | 0.26 ± 0.01                            | 3.40 ± 0.25                            | 3.12 ± 0.27                            | 0.24 ± 0.01                            |
| Tryptophan          | 0.82 ± 0.01                            | 3.96 ± 0.30                            | 4.39 ± 0.41                            | 0.36 ± 0.01                            |
| Valine              | 2.71 ± 0.09                            | 4.27 ± 0.01                            | -                                      | 0.01 ± 0.01                            |
| Phenylalanine       | 1.36 ± 0.10                            | 2.30 ± 0.01                            | -                                      | 0.01 ± 0.01                            |
| Suaeda monoica      |                                        |                                        |                                        |                                        |
| Fructosanopranose   | 0.70 ± 0.01                            | 6.32 ± 0.01                            | 4.65 ± 0.43                            | 0.80 ± 0.01                            |
| Fructose            | 0.74 ± 0.01                            | 9.18 ± 0.68                            | 0.32 ± 0.01                            | 1.48 ± 0.01                            |
| Galactonanopranose  | 0.60 ± 0.01                            | 1.20 ± 0.01                            | 28.30 ± 2.15                           | 92.50 ± 8.03                           |
| Glucose             | 117.83 ± 0.06                          | 272.49 ± 2.5                           | 45.54 ± 3.81                           | 87.70 ± 4.44                           |
| Lactose             | 0.64 ± 0.03                            | 0.49 ± 0.02                            | 2.91 ± 0.22                            | 4.92 ± 0.06                            |
| Ribon-1,4-lactone    | 1.07 ± 0.01                            | 6.59 ± 0.48                            | 1.42 ± 0.01                            | 1.43 ± 0.01                            |
| Trehalose           | 3.24 ± 0.01                            | 6.17 ± 0.58                            | 1.12 ± 0.06                            | 0.33 ± 0.02                            |
| Xylopyranose        | 0.78 ± 0.01                            | 5.51 ± 0.01                            | -                                      | 0.01 ± 0.01                            |
| Galactitol          | 2.23 ± 0.01                            | 21.03 ± 1.57                           | 17.51 ± 1.56                           | 3.38 ± 0.04                            |
| Ribonolose          | 3.30 ± 0.30                            | 13.39 ± 0.03                           | -                                      | 0.21 ± 0.00                            |
| Melibiose           | 0.19 ± 0.01                            | 5.12 ± 0.01                            | 0.49 ± 0.02                            | 2.91 ± 0.22                            |
| Methyl galactoside  | 0.22 ± 0.01                            | 1.30 ± 0.08                            | 1.61 ± 0.13                            | 0.22 ± 0.01                            |
| Scopolin            | 0.46 ± 0.01                            | 1.40 ± 0.01                            | -                                      | 0.21 ± 0.00                            |
| Sucrose             | 204.74 ± 1.21                          | 377.9 ± 0.19                           | 395.72 ± 0.2                           | 422.7 ± 2.57                           |
| Trimethylsilyl       | 0.77 ± 0.01                            | 0.98 ± 0.01                            | 1.33 ± 0.07                            | -                                      |

**Table 1. Comprehensive metabolite profiling of Suáeda spp. under different integrated abiotic stress conditions.**
| Metabolites      | Suaeda monoica | Suaeda fruticosa | Suaeda monoica | Suaeda fruticosa | Suaeda monoica | Suaeda fruticosa |
|------------------|----------------|------------------|----------------|------------------|----------------|-----------------|
|                  | Control         | Salt Drought     | Control         | Salt Drought     | Control         | Salt Drought     |
| Galacturonic acid| 1.10 ± 0.01     | 9.35 ± 0.47      | 39.39 ± 0.02    | 2.85 ± 0.02      | 3.03 ± 0.01     | 3.53 ± 0.01      |
| Mannitol         | 0.36 ± 0.02     | -                | -              | -                | -              | -               |
| Glyceraldehyde   | 1.96 ± 0.03     | 13.52 ± 1.02     | 16.57 ± 1.45    | 1.73 ± 0.01      | 1.71 ± 0.02     | 1.48 ± 0.01      |
| Threonine        | 4.30 ± 0.05     | 5.62 ± 0.01      | 6.91 ± 0.60     | 1.75 ± 0.01      | 1.23 ± 0.10     | 3.11 ± 0.28      |
| Ribonic acid     | 0.59 ± 0.04     | 7.59 ± 0.44      | 6.74 ± 0.59     | 1.30 ± 0.01      | 1.44 ± 0.01     | 1.15 ± 0.01      |
| Citric acid      | 3.06 ± 0.05     | 46.95 ± 3.40     | 39.58 ± 3.49    | 9.21 ± 0.01      | 17.27 ± 0.30    | 12.33 ± 0.15     |
| Glucuronic acid  | 0.16 ± 0.01     | 1.22 ± 0.05      | 2.50 ± 0.01     | 0.22 ± 0.01      | 3.52 ± 0.01     | 2.34 ± 0.01      |
| Psicose          | -               | 17.23 ± 0.01     | 45.89 ± 0.02    | 1.41 ± 0.01      | -              | 14.68 ± 0.06     |
| Galactaric acid  | 8.92 ± 0.14     | 44.58 ± 3.33     | 6.78 ± 0.17     | 18.29 ± 0.08     | 16.08 ± 0.12    | 27.66 ± 0.30     |
| Tartaric acid    | 10.39 ± 0.10    | 40.53 ± 3.06     | 37.43 ± 3.28    | 9.67 ± 0.06      | 10.00 ± 0.07    | 12.99 ± 0.05     |
| Oxalic acid      | 0.24 ± 0.01     | -                | 1.19 ± 0.02     | 0.11 ± 0.01      | 0.17 ± 0.01     | -               |

**Fatty acids**

| Metabolites      | Suaeda monoica | Suaeda fruticosa | Suaeda monoica | Suaeda fruticosa | Suaeda monoica | Suaeda fruticosa |
|------------------|----------------|------------------|----------------|------------------|----------------|-----------------|
|                  | Control         | Salt Drought     | Control         | Salt Drought     | Control         | Salt Drought     |
| 2-Aminobutyric acid | 0.60 ± 0.01     | 1.76 ± 0.11      | 2.95 ± 0.25     | 0.39 ± 0.01      | 0.10 ± 0.01     | 0.36 ± 0.01      |
| Butyric acid     | 0.74 ± 0.01     | 4.36 ± 0.33      | 4.20 ± 0.34     | 0.93 ± 0.01      | 0.73 ± 0.01     | 0.73 ± 0.02      |
| Glucomannanose   | -               | 9.95 ± 0.78      | 21.15 ± 1.78    | 0.83 ± 0.01      | 1.98 ± 0.05     | 1.40 ± 0.01      |
| Malic acid       | 18.12 ± 0.19    | 205.7 ± 15.6     | 301.8 ± 26.3    | 27.15 ± 0.03     | 26.48 ± 0.06    | 32.37 ± 0.05     |
| Myo-Inositol β−  | 0.76 ± 0.01     | 7.81 ± 0.58      | 7.81 ± 0.64     | 0.48 ± 0.01      | 1.10 ± 0.01     | 1.35 ± 0.01      |
| Galactopyranoside| 0.63 ± 0.02     | 2.01 ± 0.01      | -              | 1.10 ± 0.01      | 0.96 ± 0.01     | 0.78 ± 0.01      |

**Miscellaneous**

| Metabolites      | Suaeda monoica | Suaeda fruticosa | Suaeda monoica | Suaeda fruticosa | Suaeda monoica | Suaeda fruticosa |
|------------------|----------------|------------------|----------------|------------------|----------------|-----------------|
|                  | Control         | Salt Drought     | Control         | Salt Drought     | Control         | Salt Drought     |
| 2-Butenediac acid | 0.13 ± 0.01     | 1.39 ± 0.10      | 2.03 ± 0.01     | 0.43 ± 0.01      | 0.38 ± 0.01     | 0.16 ± 0.01      |
| 3-Hydroxy-4,5-   | 0.47 ± 0.01     | 7.06 ± 0.53      | 25.32 ± 2.21    | 1.57 ± 0.01      | 1.54 ± 0.04     | 3.48 ± 0.07      |
| Tyrosine         | 0.50 ± 0.01     | 2.09 ± 0.18      | 3.11 ± 0.01     | 0.20 ± 0.01      | 0.34 ± 0.01     | 0.21 ± 0.01      |
| Ethanolamine     | 0.08 ± 0.01     | 1.92 ± 0.16      | 3.14 ± 0.01     | 0.36 ± 0.01      | 0.53 ± 0.01     | 0.55 ± 0.01      |
| Ferric acid      | 0.36 ± 0.01     | 2.39 ± 0.19      | -              | 0.34 ± 0.01      | 0.40 ± 0.01     | 0.55 ± 0.02      |
| Melatonin        | 36.72 ± 0.75    | 198.8 ± 13.6     | 527.2 ± 45.7    | 63.54 ± 0.40     | 71.22 ± 1.27    | 176.1 ± 9.71     |
| Quinic acid      | 0.24 ± 0.01     | 1.01 ± 0.08      | 2.44 ± 0.01     | 0.26 ± 0.01      | 0.38 ± 0.01     | 0.19 ± 0.01      |
| Silanol          | 3.25 ± 0.07     | 15.67 ± 1.18     | 9.60 ± 0.84     | 1.58 ± 0.01      | 1.19 ± 0.01     | 2.46 ± 0.01      |
| Uridine          | 0.14 ± 0.01     | 1.82 ± 0.14      | 1.18 ± 0.01     | 0.39 ± 0.01      | 0.23 ± 0.01     | 0.17 ± 0.01      |
| Flavonoid        | -               | -                | -              | -                | -              | -               |
| Kaempferol       | -               | -                | -              | -                | -              | -               |

Note: -, not detected or trace amount. Metabolite concentration is expressed as mean ± SE (n = 3) µg/g fresh weight.
Amino acids, including proline, serine, threonine, aspartic acid, glutamic acid, glutamine, and tryptophan, accumulate differentially in both *Suaeda* species under salt and drought stress under ambient CO$_2$ conditions. In contrast, under elevated CO$_2$ conditions, their concentrations decreased under salt or drought stress compared to the corresponding control conditions, but the overall level was higher than ambient CO$_2$ conditions. Asparagine, valine, and phenylalanine were not detected in *S. fruticosa* under ambient CO$_2$ (control and stress) conditions. However, these amino acids were detected under elevated CO$_2$ (control and stress; salt or drought) conditions in *S. monoica*. Some photorespiratory essential amino acids, such as serine and glycine, were also detected in both *Suaeda* species. We found that the serine concentration was higher in *S. monoica* compared to *S. fruticosa* under different stress conditions. Interestingly, their concentrations decreased under elevated CO$_2$ stress conditions compared to their respective control conditions.

Sugars are also major metabolites contributing to stress tolerance. About 20 different sugars were detected under varying stress conditions. Sugars are an essential energy source for any biosynthesis process, including lipids and proteins, and act as a vitamin C precursor. Furthermore, sugars, also known as osmoprotectants, are commonly accumulated under stress conditions in plants. One of the sugars (i.e., fructose) was detected in both *Suaeda* species under ambient CO$_2$ (control and stress) conditions but not under elevated CO$_2$ conditions. Some important sugars, including galactose, turanose, rhamnose, melibiose, cellobiose, sucrose, lactose, galactinol, lactose, galactinol, and ribono-1,4-lactone, were detected in both *Suaeda* species. High accumulation was determined under stress conditions, but their contents were not significantly affected under elevated CO$_2$ stress conditions.

Four sugar acids, including riboninic acid, galacturonic acid, glyceric acid, and threonic acid, were detected in both *Suaeda* species under salt and drought stress at ambient or elevated CO$_2$ stress conditions. These sugar acids accumulated under ambient CO$_2$ stress conditions compared to control, whereas their content decreased under elevated CO$_2$ stress conditions. Likewise, other sugar acids and metabolites of the TCA cycle, such as citric acid, tartaric acid, glucuronic acid, oxalic acid, psicose, and galactaric acid, showed a similar trend. Aside from these metabolites, nine miscellaneous metabolites, including 2-butanedioic acid, 3-hydroxy-DL-tyrosine, erythrono, ethanolamine, ferulic acid, melatonin, quininic acid, silanol, and uridine, were detected in both species under control and stress conditions (Table 1). Two of the most important bioactive compounds that act as antioxidants and anti-diabetics (melatonin and quininic acid) accumulated in ambient CO$_2$ stress conditions, especially under drought stress in both *Suaeda* plants. In contrast, their accumulation decreased under elevated CO$_2$ stress conditions compared to control in *S. fruticosa*.

### 3.2. Multivariate Statistical Analysis

The integrated PCA showed the possible correlation of plant responses to different stress conditions. A bi-plot deduced from the PCA separated treatments into the first two components with an overall variability of 84.91% (PC1: 75% and PC2: 9.91%). The bi-plot clustered metabolites according to the effects of stress treatments. The most significant effects were observed with elevated CO$_2$ among all other stress combinations in both *Suaeda* species (Figure 1). Similarly, a score plot was generated based on PLS-DA. The score plot represented a clustering of different metabolites detected in plants treated with different stress conditions (Figure 2). The score plot revealed that drought and salt stress significantly affect the metabolism of *S. monoica* compared to other stress conditions, whereas in *S. fruticosa*, elevated CO$_2$ had a significant effect on plants compared to other stresses. OPLS-DA is a regression model used to calculate a correlation between the multivariate data and a response variable in the metabolomics. OPLS-DA of *S. monoica* and *S. fruticosa* showed a correlation between two plants treated with different stress conditions. The $R^2$ and $Q^2$ values revealed that the model is more stable, reliable, and excellent for the study. A hierarchical cluster analysis was performed and heatmaps generated based on Spearman's rank correlation coefficients showed a correlation among pairs of plants.
treated with varying stress conditions and pairs of extracted metabolites. Spearman's rank correlation analysis is commonly used to study a relationship between two variables. In this correlation study, we determined whether two variables covary (vary together with another variable). A cumulative heatmap showing a correlation between metabolites and plants grown under different stress conditions (Figure 3) revealed differential expression or accumulation of metabolites under stress conditions.

Figure 1. Cont.
Figure 1. An integrated bi-plot-based principal component analysis. Bi-plot-correlated metabolites expressed in response to stress conditions in (A) *S. monoica* and (B) *S. fruticosa*. C: plants grown under ambient conditions; EC: plants under elevated CO₂ conditions; S: salt (500 mM NaCl) stress under ambient CO₂ conditions; SE: salt (500 mM NaCl) stress under elevated CO₂; D: drought or osmotic (5% PEG) stress under ambient CO₂ conditions; DE: drought or osmotic (5% PEG) stress under elevated CO₂ conditions.
**Figure 2.** Partial least squares discriminant analysis (PLS-DA) of plants treated with different stress conditions. The PLS-DA represents the important metabolites and their relative accumulation in (A) *S. monoica* and (B) *S. fruticosa* in response to different stress conditions based on the variable importance in projection score. C: plants grown under ambient conditions; EC: plants under elevated CO$_2$ conditions; S: salt (500 mM NaCl) stress under ambient CO$_2$ conditions; SE: salt (500 mM NaCl) stress under elevated CO$_2$; D: drought or osmotic (5% PEG) stress under ambient CO$_2$ conditions; DE: drought or osmotic (5% PEG) stress under elevated CO$_2$ conditions.

**Figure 3.** Heatmap representing the correlations between metabolites and plants treated with different stress conditions. The heatmap represents the correlation between metabolites and (A) *S. monoica* and (B) *S. fruticosa* grown under varying stress conditions. C: plants grown under ambient conditions; EC: plants under elevated CO$_2$ conditions; S: salt (500 mM NaCl) stress under ambient CO$_2$ conditions; SE: salt (500 mM NaCl) stress under elevated CO$_2$; D: drought or osmotic (5% PEG) stress under ambient CO$_2$ conditions; DE: drought or osmotic (5% PEG) stress under elevated CO$_2$ conditions.
In ambient CO₂ conditions, intermediates of the Krebs cycle, including malic acid, accumulate in leaves of *S. monoica* under salt and drought stress conditions, whereas in *S. fruticosa*, no significant difference was found between control and stress conditions. In contrast, in elevated CO₂ conditions, the malic acid concentration was decreased under stress conditions compared to control conditions in both *Suaeda* species. This study demonstrated that the key metabolites involved in plant metabolism were differentially accumulated or expressed under varying stress conditions in *Suaeda* plants (Figure 4). These metabolites are involved in basic metabolic pathways.

![Diagram](image_url)

**Figure 4.** Schematic presentation of the pathways for certain important metabolites. Metabolites were differentially accumulated or expressed under varying stress conditions. The relative quantification of metabolites was performed by GC-MS. Samples were analyzed and the average metabolite abundance was used to calculate the differences of the changes (represented by different colors) relative to control. C: plants grown under ambient conditions; EC: plants under elevated CO₂ conditions; S: salt (500 mM NaCl) stress under ambient CO₂ conditions; SE: salt (500 mM NaCl) stress under elevated CO₂; D: drought/osmotic (5% PEG) stress under ambient CO₂ conditions; DE: drought/osmotic (5% PEG) stress under elevated CO₂ conditions.

### 4. Discussion

Plants have different strategies to deal with stresses, including adjusting their metabolic status [14]. Plants under individual or a combination of salt, drought, and elevated CO₂ stress conditions are coordinated with the activation of different molecular and physiological responses. However, these changes lead to alterations in plant metabolism that mitigate the damaging effects of stress combinations. The acclimatization of plant species to any stress seems to be diverse from a metabolic point of view. Different stresses induce differential gene expression and modifications in various metabolites, including amino acids, organic acid, and carbohydrates, which play essential roles in carbon assimilation, photorespiration, signal regulation, and protein synthesis [80,81]. Many studies have demonstrated that elevated CO₂ could promote plant metabolism and ameliorate the detrimental effects of abiotic stress on plant species varieties. In C₃ plants, elevated CO₂ decreases ROS production and oxidative damage; however, this means that other non-stomatal processes and reduced photorespiration contribute to stress mitigation [82].
Omics methodologies widely used to study plant responses provide multiple metabolic progressions under elevated CO$_2$ stress conditions [10,53,83]. Therefore, we speculated that although these two halophytes belong to the same genus (but different species), their strategies to deal with different stresses may be quite different.

Metabolite profiling is an effective and quantitative process used to elucidate the stress tolerance mechanism. Plants under different stress combinations (salt, drought, and elevated CO$_2$) have revealed a wide variety of metabolites that precisely change during stress, such as osmoprotectants, amino acids, fatty acids, carbohydrates, sugar acids, and some intermediates of the Krebs cycle [84,85]. The accumulation of osmoprotectants, such as compatible solutes (proline and sucrose), is a typical plant response involved in several mechanisms, such as maintaining the membrane’s protein stability and cell osmotic pressure (adjustment) [86]. Proline accumulated under salt and drought stress under ambient CO$_2$ conditions in both _Suaeda_ species. In contrast, under elevated CO$_2$ (control and stress) conditions, the proline concentration significantly increased compared to ambient CO$_2$ control and stress conditions. Likewise, sucrose accumulation was considerably higher among other metabolites during stress conditions. Sucrose accumulated under salt and drought stress conditions in both _Suaeda_ species in ambient CO$_2$ conditions as compared to control (Table 1). Similar results were also observed in _Arabidopsis thaliana_ under drought and heat stress conditions, where proline accumulated during drought stress, whereas sucrose accumulated during a combination of stresses to protect the mitochondrial and cellular components during stress [87]. In contrast, purslane plants accumulated proline under individual, heat, and drought stress conditions but not in combination [88]. In ambient CO$_2$ conditions under heat stress, different metabolites were significantly accumulated, such as succinic acid, aspartic acid, some essential amino acid, malic acid, and some sugars in _Poa pratensis_ (Kentucky bluegrass) [89].

An accumulation of other metabolites and their concentrations was also observed in _Suaeda_ species exposed to salt and drought stress with a combination of ambient and elevated CO$_2$ conditions (Figures 1–3). In this study, _Suaeda_ species can cope with the adverse effects of abiotic stress conditions by accumulating different sugars, amino acids, fatty acids, and other metabolic compounds. Furthermore, different amino acids, including asparagine, citramalic acid, glycine, alanine, glutamic acid, aspartic acid, glutamine, norvaline, proline, serine, threonine, valine, tryptophan, and phenylalanine, showed increased accumulation under ambient CO$_2$ stress conditions compared to controls. In _S. monoica_, asparagine content increased under salt and drought stress, supporting the observation that accumulation is up to a substantial extent under stress conditions [90], implying that certain amino acids have different sensitivity toward stress responses in different plants under different stress conditions.

Similarly, _A. thaliana_ and purslane plants accumulate different types of amino acids, including tyrosine, tryptophan, glutamine, valine, and ornithine, to play an essential role in the osmotic adjustment of cellular components aimed at maintaining leaf turgor under stress treatment [83,84]. Aromatic amino acids, such as tryptophan, involved in the downstream regulation of the shikimic acid pathway increased in both _Suaeda_ species under salt and drought stress in ambient CO$_2$ conditions. Furthermore, aromatic amino acids were decreased in both _Suaeda_ species under salt and drought stress in elevated CO$_2$ conditions as compared to control plants (Table 1). Accumulation of important amino acids under stress could be correlated with protein synthesis to protect and initiate fast recovery after stress in plant metabolism and osmotic adjustments [91]. This observation indicated that salt and drought stress rigorously affected C$_4$ _Suaeda_ physiological constraints, and that these detrimental effects were alleviated by elevated CO$_2$ when combined with salt and drought stress.

In addition to amino acids, an increase in carbohydrates within a cell may be another strategy used for osmotic adjustment under stress conditions. Certain soluble sugars, including fructose, glucose, and sucrose, increased in _Thellungiella_ and _Arabidopsis_ [92]. The sucrose content in the xero-halophyte species, _Atriplex halimus_, increased significantly under
salt stress conditions [93]. In this study, different sugar molecules accumulated in the leaves of both Suaeda species, however the accumulation was found to be insignificant, demonstrating the efficient protection of membranes and photosystem II from photo-oxidation damages. Koussevitzky et al. [94] reported that during a combination of high temperature and drought stress, accumulated malic enzyme in A. thaliana was correlated with its increased activity and a decline in malate and oxaloacetate concentrations, whereas under elevated CO$_2$ conditions, a C$_4$ turf grass species (Festuca arundinacea) showed improved abiotic stress tolerance under a combination of drought and heat stress by improving plant water use, photosynthesis efficiency, cellular membrane stability, and reduced rate of photorespiration [95]. Hence, it was considered that the metabolic alteration associated with the mitigation of abiotic stress damage by elevated CO$_2$ would offer further insights into the collaborative effects of salt stress, drought stress, and enhanced environmental CO$_2$ concentrations in plant species.

Thus, different bioactive compounds, such as quinic acid, kaempferol, and melatonin, were present in both Suaeda species in precise concentrations. Melatonin is a pleiotropic metabolic compound that not only plays a role in antioxidant activity but also induces the regulation of gene expression in various physiological progressions, including plant growth [96], germination, rooting [97], photosynthesis [98], and osmoregulation [99], and protects against different abiotic and biotic stresses [100–102]. Recently, the melatonin receptor was first identified and characterized in Arabidopsis [103]. Interestingly, different studies have indicated that melatonin might be considered as an emerging phytohormone, and its multiple functions also include being a vital redox homeostasis regulator in plant systems [104,105]. In this study, the melatonin content was higher in stress conditions in both Suaeda plants under ambient CO$_2$ conditions but decreased under elevated CO$_2$ stress conditions. Previous research suggested that kaempferol and quinic acid possess potent antioxidant and antidiabetic properties [106–108]. Kaempferol is a natural flavonoid that shows antioxidant, anticancer, and anti-inflammatory therapeutic properties and is present in different plant species [78,109].

Similarly, the ethanolic extract from Calotropis procera leaves was analyzed using ultra-high performance liquid chromatography quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS/MS). The extract also showed higher rates of antidiabetic and antioxidant metabolites, such as quinic acid, kaempferol, and p-hydroxybenzoic acid [108]. Kaempferol identified from S. maritima leaf extracts showed cytotoxic activity against human tumor cell lines [110]. Different types of metabolites accumulate in different species to provide osmotic protection under varying stress conditions. Differential synthesis and accumulation of metabolites are associated with enhanced stress tolerance [111], and may be result of the various pathways initiated in the different species in response to varying stresses.

This study addressed the variability of the metabolites in halophytes using metabolomics and analyzed their effects on stress tolerance, and also confirms that Suaeda is a valuable source of bioactive compounds with significantly potent antioxidant effects. Overall, this study suggests that differential accumulation of various metabolites of C$_4$ Suaeda in response to different stress conditions, such as salt, drought, and elevated CO$_2$ stress conditions, provides insights into stress tolerance in C$_4$ plant species in relation to climate change. This work provides new perspectives on the important secondary metabolite pathway involved in stress tolerance between Suaeda species. Consequently, a detailed study of the tolerance mechanism of wetland plants is still needed.

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