

Physiological Responses of *Tamarix ramosissima* to Extreme NaCl Concentrations

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ABSTRACT

Hydrologic alterations of river systems in western North America over the past century have increased soil salinity, contributing to the establishment and spread of an introduced halophytic species, *Tamarix ramosissima* (Ledeb.). The physiological responses of *Tamarix ramosissima* to salinity stress are incompletely known. To assess the salinity tolerance of this species, we measured several whole plant and leaf-level physiological responses of *Tamarix ramosissima* cuttings grown in a controlled environment over three NaCl concentrations (0, 15 and 40 g·L⁻¹). *Tamarix ramosissima* photosynthesis (A_{2000}), stomatal conductance to water (g_s), water potential (Ψ_w), and the maximum quantum yield of photosystem II (F_v/F_m) decreased at 15 and 40 g·L⁻¹ NaCl compared to control treatments. However, after approximately 35 days, *Tamarix ramosissima* had increased photosynthetic rates, maximum quantum yield of photosystem II, and stomatal conductance to water. These data suggests that physiological functioning of *Tamarix ramosissima* acclimated to extremely high NaCl concentrations over a relatively short period of time. Additionally, we present preliminary evidence that suggests proline synthesis may be the mechanism by which this species adjusts osmotically to increasing salinity.

Keywords: Chlorophyll Fluorescence, Gas Exchange, Proline, Saltcedar, Salt Stress, Tamarisk

1. Introduction

Many adaptations have been hypothesized as mechanisms facilitating the spread of the invasive, exotic tree species, *Tamarix ramosissima* Ledeb., along disturbed riparian corridors in western North America. These include high seed production, high growth rates [1], drought tolerance [2], ability to resprout after fire [3] or grazing, and the facultative phreatophytic nature of the species [4]. In western North America, riparian soils are naturally saline from low annual precipitation, but salinization has been exacerbated by river flow regulation, groundwater pumping, and river channel changes that decrease the frequency of overbank flooding [5-7]. The halophytic nature of the species is also commonly hypothesized as a primary factor contributing to the spread and establishment of *Tamarix ramosissima* [8-11].

Although salinity adversely affects the production and growth of most species, halophytes are adapted to tolerate highly saline environments. One mechanism to tolerate high salinities is to regulate Na⁺ transport to shoots

and leaves [12,13]. Salts can be excluded from leaves by selective uptake by root cells, although it is unclear which cell types control this selectivity [14]. Some halophytic species have specialized salt glands or salt bladders that exude salt from the plant via apoplastic pathways [15,16]. Additionally, compartmentalization and the synthesis of compatible solutes are also important salt tolerating mechanisms. Many halophytes compartmentalize Na⁺ in cell vacuoles to limit toxicity in the cytoplasm [14,17-19]. Compartmentalization of Na⁺ disrupts the osmotic balance in cells between the vacuole and cytoplasm. Plants may synthesize compatible solutes (e.g., proline, glycine betaine) in the cytoplasm to reestablish osmotic balance. These low-molecular-mass compounds do not interfere with normal biochemical reactions [20]. However, compatible solutes are energetically expensive, requiring as much as 52 ATP per mol for synthesis [21].

Tamarix ramosissima has various salinity tolerance mechanisms. Most notably, *Tamarix ramosissima* develops salt glands that secrete excess salts that would be

accumulated by non salt-tolerant species [22]. Salt is excreted in solution through specialized salt glands via an apoplastic pathway to alleviate metabolic stress caused by Na^+ [23]. *Tamarix ramosissima* also accumulates compatible solutes during periods of salinity stress. Studies conducted along the Tarim River, China [24,25] and the Yellow River, China [26] suggest *Tamarix ramosissima* accumulates compatible solutes (proline and soluble sugars) during salinity stress to maintain internal osmotic balance. Solomon *et al.* [27] also showed that *Tamarix jordanis* Boiss. synthesizes N-methyl-L-proline (MP) and N-methyl-*trans*-4-hydroxy-L-proline (MHP) in the presence of high NaCl content. Both solutes are effective at maintaining the carboxylating activity of Rubisco.

Although *Tamarix ramosissima* has salt-tolerating mechanisms, physiological responses of *Tamarix ramosissima* to salinity stress are incompletely known and few studies have reported how increasing salinity impacts these responses. Kleinkopf and Wallace [11] reported increased salt concentrations had a marginal effect on the net exchange rates of carbon and water in *Tamarix ramosissima*. Kleinkopf and Wallace [11] also measured a decrease in *Tamarix ramosissima* growth as salinity increased, which they attributed to the increased energy required for pumping salts from leaf glands. Glenn *et al.* [8] grew a mix of shrubs and trees, including *Tamarix ramosissima*, in a greenhouse and subjected plants to a salinity gradient from 0 to $32 \text{ g}\cdot\text{l}^{-1}$ NaCl. *Tamarix ramosissima* transpiration decreased markedly between 16 and $32 \text{ g}\cdot\text{l}^{-1}$ NaCl, but growth rate showed only a minor reduction (2%).

To address this gap in our understanding of the physiological responses of *Tamarix ramosissima* to soil salinity, we measured several whole plant and leaf-level physiological responses of cuttings grown at three NaCl concentrations in a controlled environment. Using these results, we address the effects of the NaCl concentrations tested (0, 15 and $40 \text{ g}\cdot\text{l}^{-1}$) in reducing gas exchange rates, leaf water potentials, and chlorophyll fluorescence.

2. Materials and Methods

2.1. Experimental Design and Procedures

Branch tip cuttings of *Tamarix ramosissima* were collected from trees growing at two sites: the Ashland Research Site (ARS) adjacent to the Cimarron River near Ashland, Kansas, USA ($37^{\circ}11'\text{N}$ and $99^{\circ}46'\text{W}$) and the Cedar Bluff Reservoir (CBR) near Ellis, Kansas, USA ($38^{\circ}48'\text{N}$ and $99^{\circ}43'\text{W}$). Cuttings were kept moist, cut at the stem base (approximately 0.6 cm in diameter) and auxin was applied to promote root development. Cuttings were propagated in a Conviron (Pembina, North Dakota,

USA) growth chamber at Kansas State University (Manhattan, Kansas, USA) in plastic nursery pots (19.3 cm diameter, 17.8 cm deep). Prior to transplanting cuttings to pots, soils were soaked in a nutrient solution made up of 20% nitrogen 20% phosphoric acid, 20% soluble potash, 0.02% boron, 0.05% chelated copper, 0.15% chelated iron, 0.05% chelated manganese, 0.0009% molybdenum, and 0.05% chelated zinc. Pots contained 550 g of a mixture of potting soil and native soil (1:1 v/v). Native soils were collected from both the Ashland research site and Cedar Bluffs Reservoir. Controlled environment conditions were set on a 12-hour photoperiod.

NaCl was added to distilled water to make solutions of 0, 15, and $40 \text{ g}\cdot\text{l}^{-1}$ NaCl. Salinity trials were initiated by irrigating pots with 400 ml of NaCl solution over a four day period (100 ml per day) to reduce salinity shock on the cuttings. Physiological responses were measured bi-weekly on each cutting, after the total solution was added. Measurements continued until all plants within the $40 \text{ g}\cdot\text{l}^{-1}$ treatment were dead, which varied between 65 - 75 days. A total of 48 cuttings were used in the experiment. The control treatment contained 12 cuttings, whereas the 15 and $40 \text{ g}\cdot\text{l}^{-1}$ treatments contained 18 cuttings each. *Tamarix ramosissima* cuttings collected from both sites were assigned to treatments at random.

2.2. Plant Physiology

Gas exchange measurements were taken using a Licor-6400 infra-red gas analyzer with a red/blue light source and a CO_2 injector (Licor Inc., Lincoln, Nebraska, USA). Irradiance inside the cuvette was $2000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, CO_2 concentration was 400 ppm and the relative humidity was maintained at ambient. Measurements reported include photosynthetic rate at $2000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (A_{2000}), stomatal conductance to water vapor (g_s), and intercellular CO_2 concentration (C_i). Projected leaf area within the gas exchange cuvette was estimated using a Licor 3100 leaf area meter. Water potentials were measured using a Scholander pressure bomb (PMS Instruments, Albany, Oregon, USA) and the maximum quantum yield of photosystem II (F_v/F_m) was measured using a chlorophyll fluorometer (Walz Instruments, Germany). The last bi-weekly measurements before death were analyzed for each cutting using a mixed-effects model ANOVA in SAS 9.1. (Cary, North Carolina, USA). NaCl concentration was treated as a fixed effect in the model whereas date of sampling was considered a random effect to account for repeated measures in the experimental design.

2.3. Stable Isotope Analysis

On each sampling date, approximately 1g of leaf sample was collected from each cutting. Each sample was dried for 48 hours at 60°C . Samples were ground with liquid

nitrogen and then analyzed for their stable carbon isotopic signature ($\delta^{13}\text{C}$) using a Finnigan Delta-plus continuous flow isotope ratio mass spectrometer connected to an elemental analyzer. Within run precision was $<0.04\%$ for $\delta^{13}\text{C}$, while between run variation was $<0.12\%$ for $\delta^{13}\text{C}$.

2.4. Proline Determination

Free proline was determined spectrophotometrically following methods from Bates *et al.* [28]. A standard curve was generated using *L*-Proline. Approximately 0.5 g of plant material was homogenized in 10 ml of 3% sulfosalicylic acid. The homogenate was filtered through Whatman #2 filter paper and then reacted with 2 ml acid-ninhydrin and 2 ml of glacial acetic acid for 1 hour at 100°C in a test tube. The reaction was stopped by placing test tubes in an ice water bath and then mixing vigorously with toluene. The chromophore containing toluene was separated and absorbance read at 520 nm using toluene as a blank. To react at least 0.5 g of plant material with 3% sulfosalicylic acid required us to use all leaf tissues from all samples per salinity treatment by sampling date.

3. Results

Leaf-level gas exchange measurements suggest *Tamarix ramosissima* physiological functioning varied as a function of salinity (**Figure 1**). Photosynthetic rates ranged from 0.2 to $37 \mu\text{mol CO}_2 \text{ m}^{-2}\cdot\text{s}^{-1}$ among all treatments. Photosynthesis declined by 50% between control and the $40 \text{ g}\cdot\text{l}^{-1}$ NaCl treatment, but did not vary significantly by salinity treatment ($p = 0.30$, **Figure 1(a)**). Stomatal conductance to water vapor ranged from 0.01 to $0.48 \text{ mol H}_2\text{O m}^{-2}\cdot\text{s}^{-1}$ among treatments. Stomatal conductance values significantly declined nearly 75% from $0 \text{ g}\cdot\text{l}^{-1}$ NaCl concentration to $40 \text{ g}\cdot\text{l}^{-1}$ NaCl concentration ($p < 0.05$; **Figure 1(b)**). Leaf-level stomatal conductance and photosynthetic rates were lower at the $15 \text{ g}\cdot\text{l}^{-1}$ NaCl concentration compared to the control, but did not vary significantly (**Figures 1(a), (b)**).

Decreases in the maximum quantum yield of photosystem II (F_v/F_m) suggest *Tamarix ramosissima* metabolic functioning significantly declined as salinity increased from 15 to $40 \text{ g}\cdot\text{l}^{-1}$ NaCl ($p < 0.05$; **Figure 1(c)**). Mean maximum quantum yield of photosystem II for the $40 \text{ g}\cdot\text{l}^{-1}$ treatment was 0.76 ± 0.015 , whereas mean maximum quantum yield of photosystem II for control plants was 0.81 ± 0.007 . The maximum quantum yield of photosystem II ranged from 0.59 to 0.84. Ψ_w varied significantly as salinity increased ($p < 0.001$; **Figure 1(d)**). Ψ_w ranged from -0.3 to -4.0 among treatments. Mean Ψ_w values were nearly two times lower in $40 \text{ g}\cdot\text{l}^{-1}$ NaCl treatments compared to controls. Neither above-ground nor below-ground biomass were significantly affected by

salinity concentrations tested ($p > 0.05$; **Figures 1(e), (f)**). Leaf $\delta^{13}\text{C}$ significantly varied as salinity increased ($p < 0.05$; **Figure 1(g)**). Leaf $\delta^{13}\text{C}$ was most enriched in $40 \text{ g}\cdot\text{l}^{-1}$ NaCl concentration and the most depleted in control treatments. $\delta^{13}\text{C}$ values ranged from -28.1 to -36.9 among treatments.

Tamarix ramosissima physiological functioning acclimated to salinity over time (**Figure 2**). Photosynthetic rates declined immediately after initial NaCl additions, but began to increase after approximately 35 days (**Figure 2(a)**). However, of the 3 treatments, *Tamarix ramosissima* cuttings in the $40 \text{ g}\cdot\text{l}^{-1}$ NaCl treatment consistently exhibited lower photosynthesis, stomatal conductance to water, maximum quantum yield of photosystem II, and the highest proline concentrations compared to the 0 and $15 \text{ g}\cdot\text{l}^{-1}$ NaCl treatments (**Figure 2**). All plants subjected to the $40 \text{ g}\cdot\text{l}^{-1}$ NaCl concentration treatment died between 60 - 75 days after induction of the treatment.

4. Discussion

The salt tolerance of *Tamarix ramosissima* is likely one mechanism by which this species persists and expands its range in western North America compared to native riparian species [29-32]. Increasing salinity is known to cause physiological stress in most species [19,33,34], but few studies have examined the physiological responses of *Tamarix ramosissima* to salinity [8,11]. Our results are consistent with Glenn *et al.* [8], suggesting that *Tamarix ramosissima* leaf-level physiological responses decrease at extremely high NaCl concentrations. Our results also show short term acclimation to both high salinity treatments, however, growth in extreme salt concentrations ($40 \text{ g}\cdot\text{l}^{-1}$) eventually resulted in death regardless of an acclimation response.

Previous work has shown that salinity imparts both ionic and osmotic stress [18,19]; our results suggest *Tamarix ramosissima* was impacted by both at high Salinity. Osmotic stress had the greatest impact on *Tamarix ramosissima* individuals. High NaCl concentration reduced stomatal conductance and Ψ_w (**Figures 1(b), (d)**). Ψ_w is highly sensitive to saline soils such that reduced water availability can be a dominant factor determining plant responses to stress [35,36]. Even low-level salt exposure can impact plant-water relations [37,38]. It is difficult to partition alterations in physiological functioning to water stress or salt-specific effects, as these changes can be co-dependent over time. After minutes to hours, growth rates and physiological responses instantaneously decline as salinity concentrations increase. Typically there is a partial recovery after initial declines, but growth rates and physiological functioning still remain low when under salt stress [14,18,19]. These quick declines also occur in plants where KCl, mannitol, or polyethylene

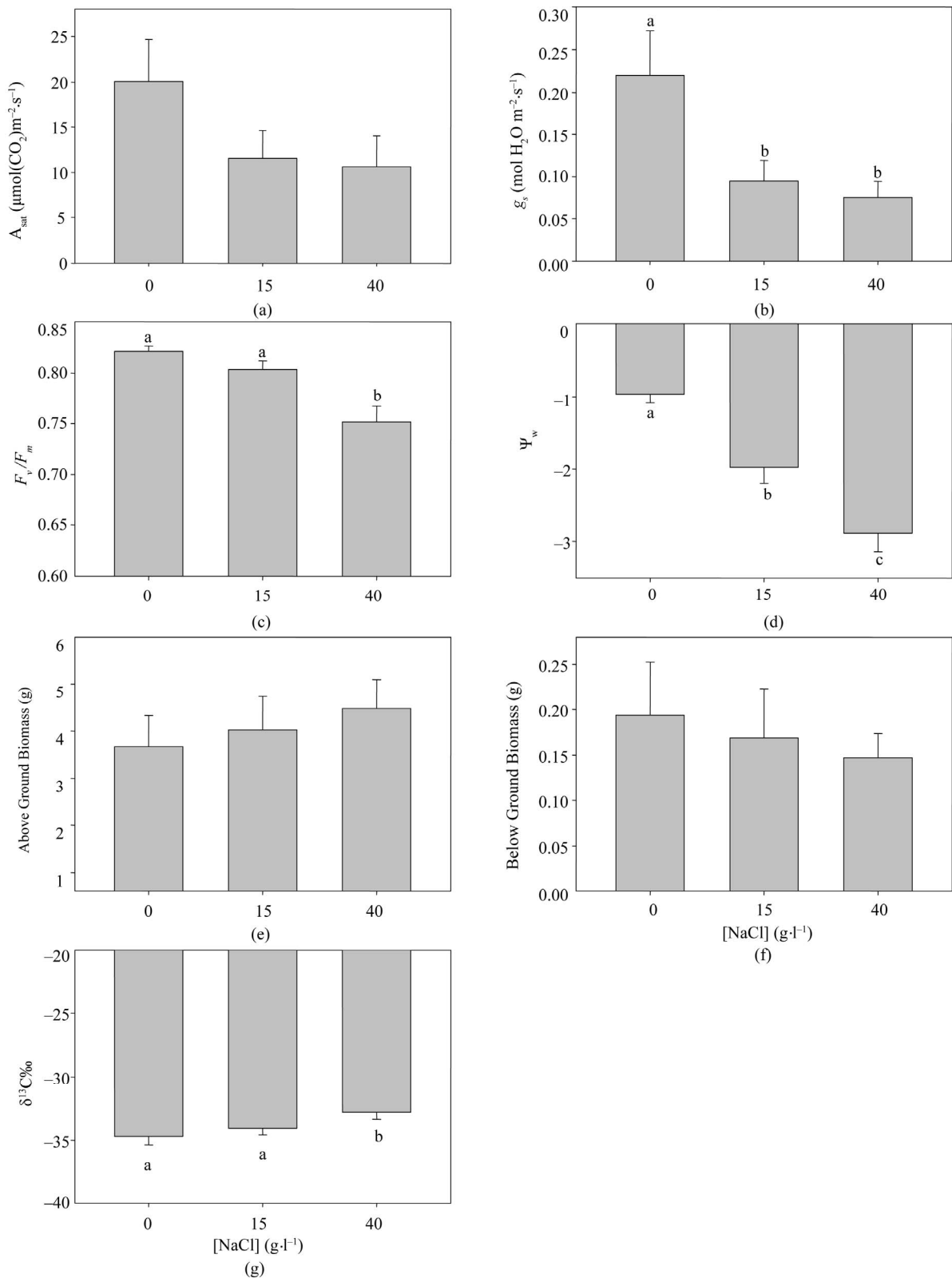


Figure 1. *Tamarix ramosissima* mean (± 1 SE) (a) photosynthetic rate at 2000 $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ ($A_{at\ 2000}$), (b) stomatal conductance to water vapor (g_s), (c) the maximum quantum yield of photosystem II (F_v/F_m), (d) water potential (Ψ_w), (e) above-ground and (f) below-ground biomass, and (g) $\delta^{13}\text{C}$ among three NaCl concentrations.

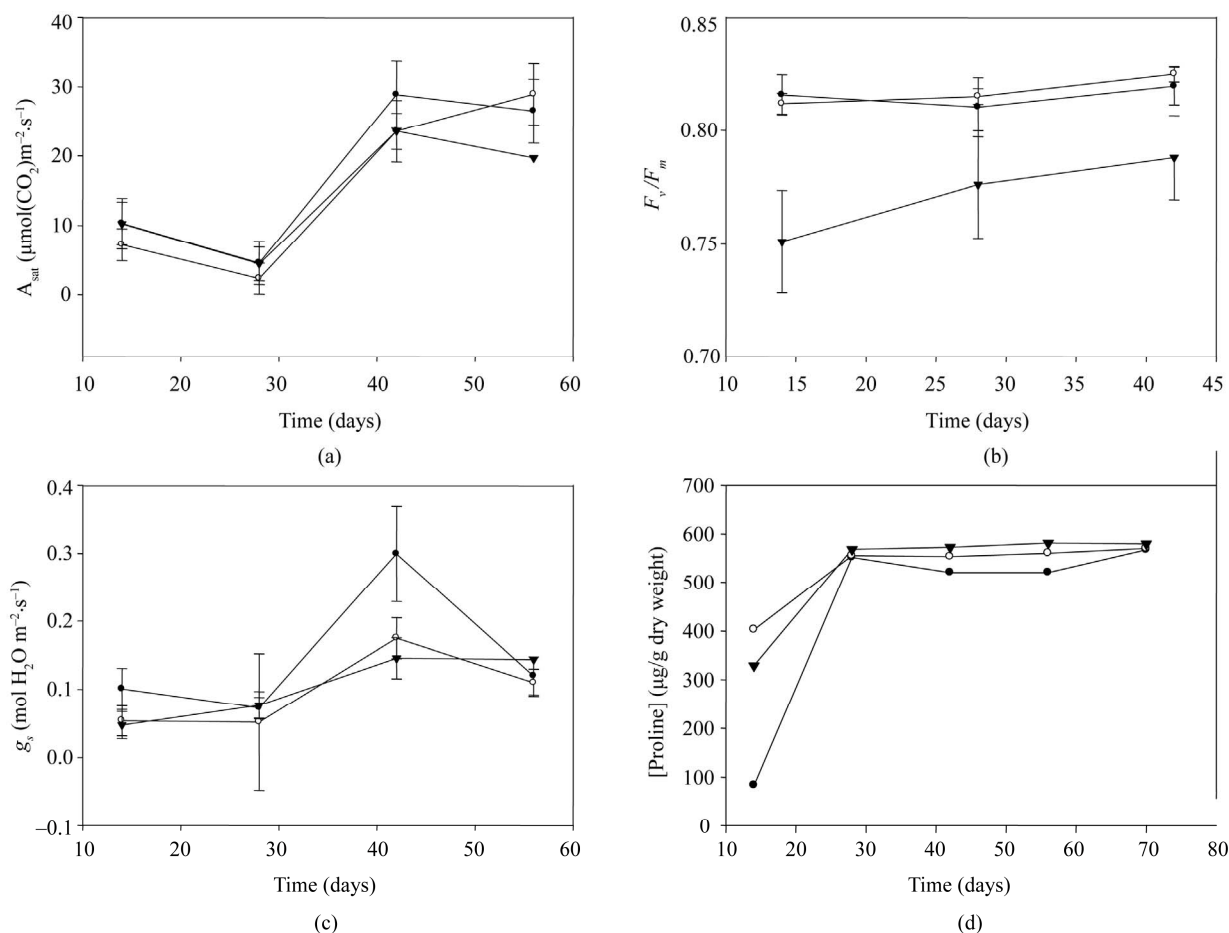


Figure 2. *Tamarix ramosissima* (a) photosynthetic rate at 2000 $\mu\text{mol m}^{-2}\cdot\text{s}^{-1}$ (A_{at2000}), (b) maximum quantum yield of photosystem II (F_v/F_m), (c) stomatal conductance to water vapor (g_s), and (d) proline concentration over time across three NaCl concentrations (closed circles = 0 $\text{g}\cdot\text{l}^{-1}$ [NaCl], opened circles = 15 $\text{g}\cdot\text{l}^{-1}$ [NaCl], closed triangles = 40 $\text{g}\cdot\text{l}^{-1}$ [NaCl]).

glycol (PEG) have been added, suggesting these responses are not solely salt-specific [20,39].

In the present study, *Tamarix ramosissima* plants subjected to 40 $\text{g}\cdot\text{l}^{-1}$ NaCl showed marked physiological declines after 14 days (Figure 2). Declines in the maximum quantum yield of photosystem II, photosynthesis, and stomatal conductance were consistent after 28 days. However, these parameters increased after 40 days. Corresponding to these increases, free proline concentration also increased in all treatments after 28 days. An increase in free proline concentration is an indicator of water stress [28,40,41]. It is possible that *Tamarix ramosissima* was able to maintain physiological functioning, including water status, by accumulating proline. Similar results have been shown for *Tamarix jordanis* [27]. It is also important to note that our high salinity treatment (40 $\text{g}\cdot\text{l}^{-1}$ or 40,000 ppm NaCl) constitutes an extreme salinity endpoint. *Tamarix ramosissima* was able to acclimate to this extreme salinity over ~35 days. The highest documented soil salinity reported for *Tamarix ramosissima* in the US

is approximately 20,000 ppm in the delta of the Colorado River where the species gives way to obligate halophytes such as *Distichlis palmeri* (Vasey) Fassett ex I.M. Johnst. [42]. The ability to acclimate to extreme salinities could provide a competitive advantage for *Tamarix ramosissima* over native glycophytes.

Proline accumulation is not the only tolerance strategy that halophytic species may utilize to maintain osmotic balance. Guard cells may be triggered to close around stomatal pores to conserve water when under osmotic stress [43,44]. The integrated stomatal behavior of leaves is commonly inferred by measuring the $\delta^{13}\text{C}$ stable isotopic signature as an estimate of water use efficiency [45]. Our results suggest high salinity reduces stomatal aperture in *Tamarix ramosissima*. Values of leaf $\delta^{13}\text{C}$ were, on average, heaviest in 40 $\text{g}\cdot\text{l}^{-1}$ treatments suggesting greatest stomatal regulation compared to 0 and 15 $\text{g}\cdot\text{l}^{-1}$ NaCl treatments. Similarly, our gas exchange data show reduced stomatal conductance at 40 $\text{g}\cdot\text{l}^{-1}$ NaCl. In controlled outdoor experiments *Tamarix ramosissima*

maintains high leaf stomatal conductance when under water or salt stress [9,46-48].

The overall objective of this study was to assess whole plant and leaf-level physiological responses of *Tamarix ramosissima* to extreme NaCl concentrations. Previous results suggested that *Tamarix ramosissima* maintained physiological functioning in the field from 0 to 14 g·l⁻¹ NaCl [47]. In this study, *Tamarix ramosissima* had decreased gas exchange, maximum quantum yield of photosystem II, and Ψ_w at 15 and 40 g·l⁻¹ NaCl, compared to the control. Physiological functioning changed over time as salinity stress was induced, suggesting short-term acclimation. Results from this study suggest that NaCl concentrations of 15 g·l⁻¹ or higher impact *Tamarix ramosissima* physiological functioning, but physiological responses may acclimate over time, even at extremely high salinities. Long-term physiological acclimation to high salinities by this species will require further assessment.

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