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ARTICLE

Salix species variation in leaf gas exchange, sodium, and nutrient parameters at three levels of salinity

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Abstract: Leaf gas exchange, Na^+, and nutrient parameters were quantified under control (CTL), medium (MST), and high (HST) saline treatments for *Salix discolor* Muhl. (DIS), *Salix eriocephala* Michx. (ERI), and *Salix interior* Rowlee (INT). Net photosynthesis (P_n) increased from CTL to MST for all species but remained unchanged between MST and HST for surviving INT plants in HST. *Salix interior* had greater water-use efficiency (WUE) than DIS and ERI. Under CTL, INT had 13× the leaf Na^+ concentration of DIS and ERI, but INT Na^+ only increased by 17% across each saline treatment, whereas DIS and ERI leaf Na^+ increased 6.0× from CTL to MST. *Salix discolor* and ERI had greater cation to Na^+ ratios than INT; however, INT ratios were stable across saline treatments, whereas the DIS and ERI ratios dropped precipitously. Across species and treatments, P_n had a significant positive relationship to total aboveground dry mass, supporting the sink regulation of P_n theory. Also, P_n showed a significant positive relationship to WUE and leaf N and to leaf Na^+, which probably reflects greater energy expenditure required to mitigate the higher levels of Na^+. The saline tolerance of INT may be due to natural selection pressure in the arid regions of the southwestern United States, where it is believed to have its evolutionary origins.

Key words: leaf nutrients, net photosynthesis, salinity tolerance, water-use efficiency, willows.

Résumé : Nous avons quantifié les échanges gazeux foliaires, le Na^+ et les paramètres nutritifs de *Salix discolor* Muhl. (DIS), *Salix eriocephala* Michx. (ERI) et *Salix interior* Rowlee (INT) soumis à des traitements salins d'intensités nulle (TEM), moyenne (TSM) et forte (TSF). La photosynthèse nette (P_n) a augmenté de TEM à TSM pour toutes les espèces, mais est demeurée inchangée entre TEM et TSF dans le cas des plants survivants de INT soumis au traitement TSF. L'efficacité d'utilisation de l'eau (EUE) de INT était plus élevée que celles de DIS et ERI. Dans le cas du traitement TEM, la concentration foliaire en Na^+ de INT était 13 fois plus élevée que celles de DIS et ERI, mais n'a augmenté que de 17 % pour chaque traitement de salinité alors que la concentration foliaire en Na^+ de DIS et de ERI du traitement TSM était 6 fois plus élevée que celle du traitement TEM. Le rapport entre les cations et le Na^+ de DIS et ERI était plus élevé que celui de INT. Toutefois, ce rapport était stable entre les traitements de salinité pour INT alors que ceux de DIS et ERI ont chuté de façon précipitée. Pour l'ensemble des espèces et des traitements, la P_n était significativement et positivement reliée à la biomasse aérienne sèche totale, ce qui appuie la théorie stipulant que P_n est associée à une régulation par les puits. De plus, P_n était significativement et positivement reliée à l'EUE, au N foliaire et au Na^+ foliaire, ce qui reflète probablement la plus grande dépense d'énergie nécessaire pour atténuer les plus hautes concentrations de Na^+. La tolérance à la salinité de INT peut être due à la pression de la sélection naturelle des régions arides du sud-ouest des États-Unis d'où on pense que cette espèce tire ses origines évolutives. [Traduit par la Rédaction]

Mots-clés : nutriments foliaires, photosynthèse nette, tolérance à la salinité, efficacité d'utilisation de l'eau, saules.

Introduction

Over the past 40 years, interest in the use of willows (*Salix* spp.) as a source of biomass for energy has increased concomitantly with the search for alternative energy sources (Zsuffa 1990; Labrecque et al. 1993; Labrecque and Teodorescu 2005; Volk et al. 2006). More recently, there has been a growing interest in using willows for various environmental applications and land reclamation, including phytoremediation and phytoextraction of contaminated soils (Bourret et al. 2005; Kuzovkina and Quigley 2005; Shanahan et al. 2007; Boyter et al. 2009; Kuzovkina and Volk 2009). Willows are widespread across the northern hemisphere, and Canada has 76 native willow species (Argus 2010). They are widely distributed and adapted to a large range of site conditions. Despite abundant species richness and ecological importance, native North American willows have received limited attention as a potential biomass resource, and little is known about their

growth on environmentally challenging sites (Mosseler et al. 2014). Land reclamation of highly disturbed areas such as the oil sands in western Canada requires the identification of well-adapted plants that tolerate higher levels of soil salinity (Renault et al. 1998; Kessler et al. 2010).

Saline stress is a relatively common environmental problem worldwide and often negatively impacts morphological, physiological, and biochemical processes (Fung et al. 1998; Cassaniti et al. 2013). In a review of the physiological mechanisms of plant saline tolerance, there are generally three distinct adaptations to salinity mechanisms: cellular osmotic stress adjustment; Na^+ or Cl^- exclusion; and tolerance of tissue to accumulated Na^+ or Cl^- (Munns and Tester 2008). Salinity can cause the accumulation of high concentrations of ions such as Na^+, Cl^-, and SO_4^{2-} (Cassaniti et al. 2013; Dong et al. 2013) and decreases in K^+ and Ca^{2+} concentrations in plant tissue, depending on the species (Valdez-Aguilar et al. 2009; Dong et al. 2013). The absorption of Na^+ occurs at the

Table 1. Willow species, population, and genotypes used from previously tested natural populations for biomass production used in salinity study.

Species	Population*	Selected genotypes	Genotype no.	Latitude (N)	Longitude (W)
Salix discolor (DIS)	Hawkesbury, ON	HAW D5	1	45°39′	74°75′
	Levis, PQ	LEV D3	2	46°78′	71°18′
	Levis, PQ	LEV D6	3	46°78′	71°18′
	Norton, NB	NOR D2	4	45°67′	65°81′
	Richmond Fen, ON	RIC D2	5	45°13′	75°82′
Salix eriocephala (ERI)	Ste. Anne de la Perade, PQ	ANN E6	1	46°56′	72°20′
	Fredericton, NB	FRE E1	2	45°94′	66°62′
	Green River, NB	GRE E1	3	47°34′	68°19′
	Norton, NB	NOR E10	4	45°67′	65°81′
	Riviere au Saumon, PQ	SAU E3	5	47°21′	70°35′
Salix interior (INT)	Ottawa, ON	LAF I5	1	45°42′	75°69′
	Roebuck, ON	LIM I3	2	44°80′	75°61′
	Long Sault, ON	LON I2	3	45°03′	74°89′
	Long Sault, ON	LON I4	4	45°03′	74°89′
	Pembroke, ON	PEM I4	5	45°50′	77°07′

*ON, Ontario; PQ, Quebec; NB, New Brunswick.

exclusion of other necessary cation macronutrients (K⁺, Ca²⁺, and Mg²⁺), thus the maintenance of high ratios of cations to Na⁺ is commonly associated with saline tolerance (Colmer et al. 2006; Dong et al. 2013). Consequently, particular attention has also been given to the macro- and micro-nutrient contents of plants growing on saline sites due to the interference of NaCl in nutrient uptake (Bohnert and Sheveleva 1998; Cassaniti et al. 2013).

Salix discolor Muhl. (DIS), *Salix eriocephala* Michx. (ERI), and *Salix interior* Rowlee (INT) are native to eastern and central Canada and appear promising as fast-growing sources of woody biomass production (Mosseler et al. 1988). *Salix interior* is also widespread in western North America, ranging from Mexico to Alaska. *Salix discolor* is commonly found in wet areas on a wide variety of disturbed sites, whereas ERI and INT are more commonly associated with fast-flowing streams and rivers in typical riparian habitats. *Salix interior* has also been observed colonizing highly disturbed oil sands mine tailings in western Canada (Mosseler and Major 2015).

Adaptations related to gas exchange are important to plant fitness and have significant growth and ecological implications for both inter- and intra-specific competition (Bazzaz 1979). Salinity can affect traits such as net photosynthesis (P_n), water use efficiency (WUE), chlorophyll content, and carboxylation efficiency (Chen et al. 2010; Li et al. 2013). Gas exchange varies widely among species, environments, and plant age (Hoddinott and Scott 1996; Zhou et al. 2011). Salinity has become a problem for plant growth in oil sands and other mining operations, as well as under heavy irrigation regimes in arid environments where high evapotranspiration can result in saline soils (Fung et al. 1998). Dryland salinity is now also widely recognized as an important problem for agriculture in western Canada (Acton and Gregorich 1995), and the potential use of willows for biomass production on these sites has been recognized (Hangs et al. 2011; Mirck and Zalesny 2015). How species gas exchange and nutrient parameters interact with salinity can determine a species' competitive fitness in saline soils.

Our goal was to examine and compare gas exchange and leaf sodium and nutrient parameters in three willow species under three salinity treatments. Our hypotheses are that salinity responses in willows will differ both among species and among genotypes within these species. We will also test the sink regulation of P_n hypothesis (Paul and Foyer 2001; Ainsworth et al. 2004) by comparing P_n with biomass data from Major et al. (2017) across species and salinity treatments. Is the observed P_n response to species and salinity effects a direct response to its effect on biomass? Our specific objectives were to (*i*) determine willow species variation in gas exchange and leaf sodium and nutrient parameters, (*ii*) examine species and genotypic salinity responses and

interactions using three levels of salinity, (*iii*) test the hypothesis of sink (biomass growth) regulation of P_n across species and salinity treatments, and (*iv*) examine P_n relationships to leaf sodium and nutrient parameters by species and salinity treatments.

Materials and methods

Plant material and growth conditions

Cuttings approximately 20 cm long were harvested from five genotypes selected from previous productivity and land reclamation studies from each of three willow species (DIS, ERI, and INT) and stored frozen at –5 °C in sealed plastic bags (Table 1). On 5 May 2015, cuttings were moved to a 4 °C cooler until 12 May when they were soaked in water at room temperature for 48 h prior to planting on 14 May. Cuttings were struck individually in plastic pots (15 cm high × 14 cm diameter) containing approximately 2 L of sand, which had been previously wetted with tap water.

The willow cuttings were grown in pots arranged on nine plastic-lined ebb-and-flow irrigation benches, each 1.5 × 2.4 m in surface area, at the Canadian Forest Service – Atlantic Forestry Centre (CFS–AFC) greenhouse in Fredericton, New Brunswick, Canada (45°52′N, 66°31′W). The greenhouses had natural day length, with temperature control set to vent at 25 °C during the day and heated to a minimum of 15 °C at night, with 60% relative humidity. Each table had four ramets of each of the 15 genotypes distributed randomly within each table. Saline treatment solutions were prepared and held in 155 L plastic tubs beneath each bench. Pumps circulated the treatment water to each table for 15 min twice daily, and water levels on the tables during each pumping cycle were maintained at a depth of approximately 3–5 cm, allowing water to be drawn by capillary action through the holes in the pot bottom. Pots were well watered at all times during the experiment.

Three random replicate tables were used for each of three salinity treatment solutions: control (CTL, no salt added), medium salinity treatment (MST, target electrical conductivity (EC) = 1.5 mS·cm⁻¹), and high salinity treatment (HST, target EC = 3.0 mS·cm⁻¹). Saline treatment solutions were initially prepared by filling the tubs with tap water and adding dissolved NaCl until the target EC levels were achieved, as determined by conductivity–temperature probes (model CS547a, Campbell Scientific, Inc., Logan, Utah, USA) in each tub attached to a CR10X data logger through an AM416 multiplexer (Campbell Scientific). Experiment-long averages of 15-min EC measurements in the tubs for each replicate table by treatment were as follows: CTL, EC = 0.400, 0.537, and 0.452 mS·cm⁻¹ for replicates 1, 2, and 3, respectively; MST, EC = 1.526, 1.653, and 1.493 mS·cm⁻¹; and HST, EC = 2.898,

Table 2. Willow gas exchange and stem moisture content traits ANOVAs, including sources of variation, degrees of freedom (df), mean square values (MS), P values, and coefficients of determination (R^2).

Source of variation	df	Net photosynthesis ($\mu mol \cdot m^{-2} \cdot s^{-1}$)		Stomatal conductance ($mmol \cdot m^{-2} \cdot s^{-1}$)		Water use efficiency		Respiration ($\mu mol \cdot m^{-2} \cdot s^{-1}$)		Stomatal limitation (%)		Stem moisture content (%)	
		MS	P value	MS	P value	MS	P value	MS	P value	MS	P value	MS	P value
Block	1	14.867	0.180	0.0582	**0.021**	1349.0	**0.001**	0.196	0.551	363.68	**<0.001**	30.5	**0.010**
Treatment (trt)	1	74.557	**<0.001**	0.0004	0.961	683.3	**<0.001**	0.599	0.300	511.45	**<0.001**	323.1	**<0.001**
Species	2	135.827	**<0.001**	0.0559	**0.008**	678.9	**<0.001**	2.373	**0.044**	15.54	0.313	30.0	**0.010**
Species × trt	3	13.17	0.195	0.0065	0.526	51.0	0.513	0.171	0.865	43.23	**0.035**	170.1	**<0.001**
Genotype (species)	10	1.287	0.997	0.0132	0.266	149.8	0.071	0.236	0.904	13.97	0.509	34.0	**<0.001**
Genotype (species) × trt	18	4.77	0.880	0.0172	0.080	128.3	0.084	1.041	**0.049**	21.70	0.165	28.0	**<0.001**
Error	35	7.95		0.010		75.0		0.540		14.83		6.45	
R^2			0.779		0.681		0.800		0.644		0.783		0.539

Note: P values <0.05 are in bold type.

3.056, and 2.815 mS·cm⁻¹. Treatment solution volume in the tubs was topped up with tap water daily, and salinity was adjusted (typically every 2–3 days) by adding stock NaCl solution (100 g·L⁻¹) to maintain targeted EC values.

Fertilizer (Plant-Prod 20:8:20; Plant-Prod Inc., Leamington, Ontario, Canada) was added to the tubs periodically from 26 May through 21 August, and a final dose of fertilizer was added on 15 September. The fertilizer product contains 20% N, 8% P, and 20% K, as well as 0.15% Mg, 0.40% Fe, 0.05% Mn, and 0.05% Zn. Specifically, a 195 g·L⁻¹ stock solution was prepared, and the following amounts were added to each tub: 50 mL of stock on 26 May and 17 June; 100 mL on 3, 9, 16, and 23 July; 150 mL on 29 July following draining, cleaning, and replenishment of the treatment tubs; 50 mL on 6, 19, and 27 August; and 100 mL on 15 September. Any contribution by fertilizer to measured EC levels was not corrected for, but simply led to a temporary slight increase in EC levels in all tubs and thus reduced the amount of salt needed subsequently to reach the target EC levels. Addition of 50 mL of the stock fertilizer solution typically increased the EC in the tub by 0.25 mS·cm⁻¹.

Gas exchange and parameter estimation

Gas exchange was measured using a LI-COR 6400XT portable gas exchange system with a CO_2 mixer and RGB light source on a 2 × 3 cm leaf cuvette (LI-COR, Lincoln, Nebraska, USA). Instrument settings were set for 25 °C, at an air flow of 250 $\mu mol \cdot s^{-1}$, a light level of 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and a CO_2 level of 400 ppm. Relative humidity (RH) was adjusted using a moistened desiccant to provide RH of approximately 65% in the sample chamber, resulting in a vapor pressure deficit (VPD) of approximately 1.0 kPa. Gas exchange measurements were measured on 10 dates from 13–24 July. Each sample was measured three times, and the mean was calculated. On each date, one genotype per species was selected and sampled from one replicate table of each treatment; this was repeated for 10 days until five genotypes of each species had been sampled on two replicate tables of each treatment. The youngest fully expanded cohort leaf was selected and enclosed in a leaf cuvette. After gas exchange measurements, the leaf was removed, and trimmed to the area that was enclosed inside the cuvette. The leaf area was quantified using a flatbed scanner and WinSeedle software (Regent Instruments Inc., Quebec). Gas exchange parameters examined were P_n, stomatal conductance (G_{wv}), respiration, and WUE, which was determined by dividing P_n by G_{wv}. Stomatal limitation was determined by the following calculation: (1 – Ci/Ca) × 100. Stem segments approximately 20 cm in length were subsampled on 20 October, weighed fresh, and kept separately bagged. All material was then dried at 65 °C for 48 h and weighed. Stem moisture content was calculated based on fresh mass and dry mass of the stem subsample.

Nutrient analysis

Leaf material from the youngest fully expanded cohorts from two replicates of each genotype × treatment was collected on 30 September, dried at 65 °C for 48 h, ground, and analyzed for elemental nutrients. The grinder was washed with ethanol between samples. The Laboratory for Forest Soils and Environmental Quality at the University of New Brunswick used standard protocols (method numbers TP-SSMA 15.3.1, 15.3.3, and 15.4 from Carter 1983). Total carbon, nitrogen, and sulfur were determined for each sample using an elemental analyzer (CNS-2000, LECO Corporation, St. Joseph, Michigan, USA).

Statistical analyses

The data were subjected to analyses of variance (ANOVA), with greenhouse treatment table (block), saline treatment, species, and genotype considered as fixed effects. Genotypes were nested within species. The following ANOVA model was used:

$$Y_{ijklm} = \mu + B_i + T_j + S_k + TS_{jk} + G_{l(k)} + TG_{jl(k)} + e_{ijklm}$$

where Y_{ijklm} is the dependent seedling trait of greenhouse treatment table i of treatment j of species k of genotype l of seedling m; μ is the overall mean; B_i is the effect of greenhouse treatment table (block) i ($i = 1, 2$); T_j is the effect of treatment j ($j = 1, 2, 3$); S_k is the effect of species k ($k = 1, 2, 3$); TS_{jk} is the interaction effect of treatment j and species k; $G_{l(k)}$ is the effect of genotype l nested within species k; $TG_{lj(k)}$ is the interaction effect of treatment j and genotype l nested within species k; and e_{ijklm} is the random error component. Effects were considered statistically significant at the $P = 0.05$ level, although individual P values are provided so that readers can make their own interpretations. The data satisfied the requirements of normality for ANOVA. The general linear model from Systat (Chicago, Illinois) was used for analysis. The biomass data used for examining the regression relationship to gas exchange results are from Major et al. (2017). Note that there were no surviving DIS or ERI plants in the HST at the time of gas exchange and nutrient measurements. As a result, there were missing cells in the complete ANOVA; thus a backward step analysis was performed for missing cells as in Major et al. (2007). As a result, degrees of freedom (df) have been reduced to reflect missing cells. Treatment initial df was 2 but was reduced by 1, resulting in final df of 1 (Table 2). Species × treatment had an initial df of 4 but was reduced by 1, resulting in final df of 3. Genotype (species) had an initial df of 12 but was reduced by 2, resulting in a final df of 10. Finally, genotype had an initial df of 24 but was reduced by 6, resulting in a final df of 18. A type III ANOVA was used. Note in the following text, we use the term "genotype" to describe genotype (species). Due to DIS and ERI expiration in HST, unless explicitly noted, when describing mean species results, we include the HST

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Fig. 1. (*a*) Net photosynthesis (P_n) (mean ± SE), (*b*) stomatal conductance, and (*c*) water-use efficiency by willow species under different salt treatments. Species: DIS, *Salix discolor*; ERI, *S. eriocephala*; INT, *S. interior*.

Fig. 2. (*a*) Respiration (mean ± SE), (*b*) stomatal limitation, and (*c*) stem moisture content by willow species under different salt treatments. Species: DIS, *Salix discolor*; ERI, *S. eriocephala*; INT, *S. interior*.
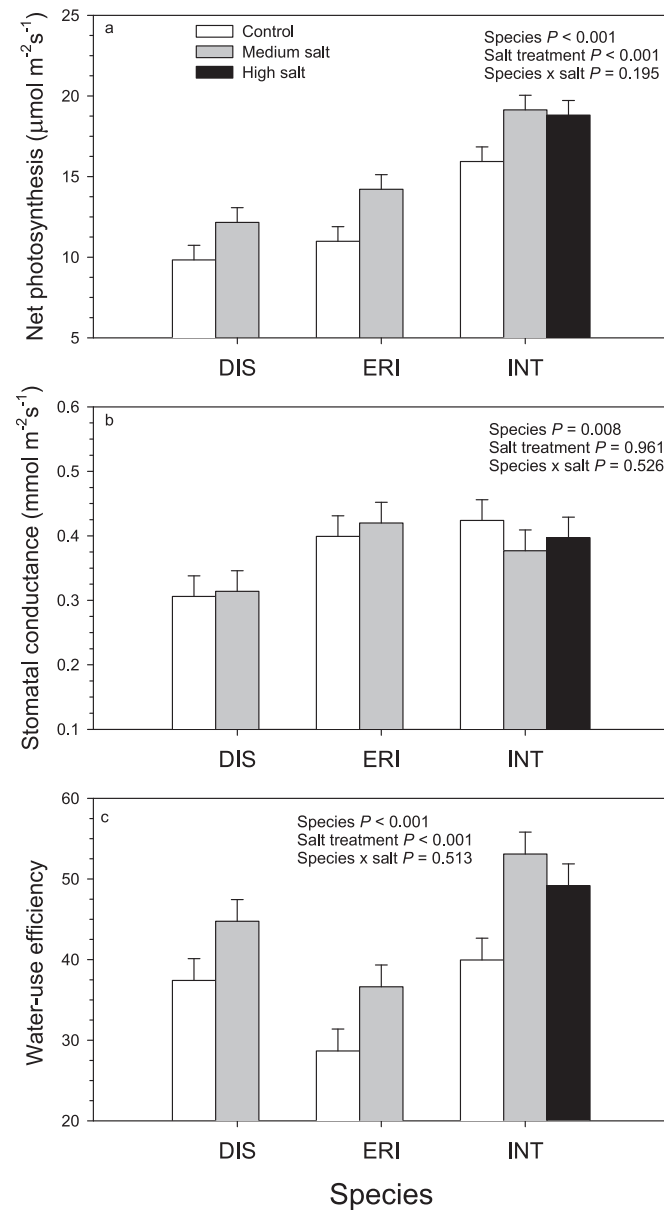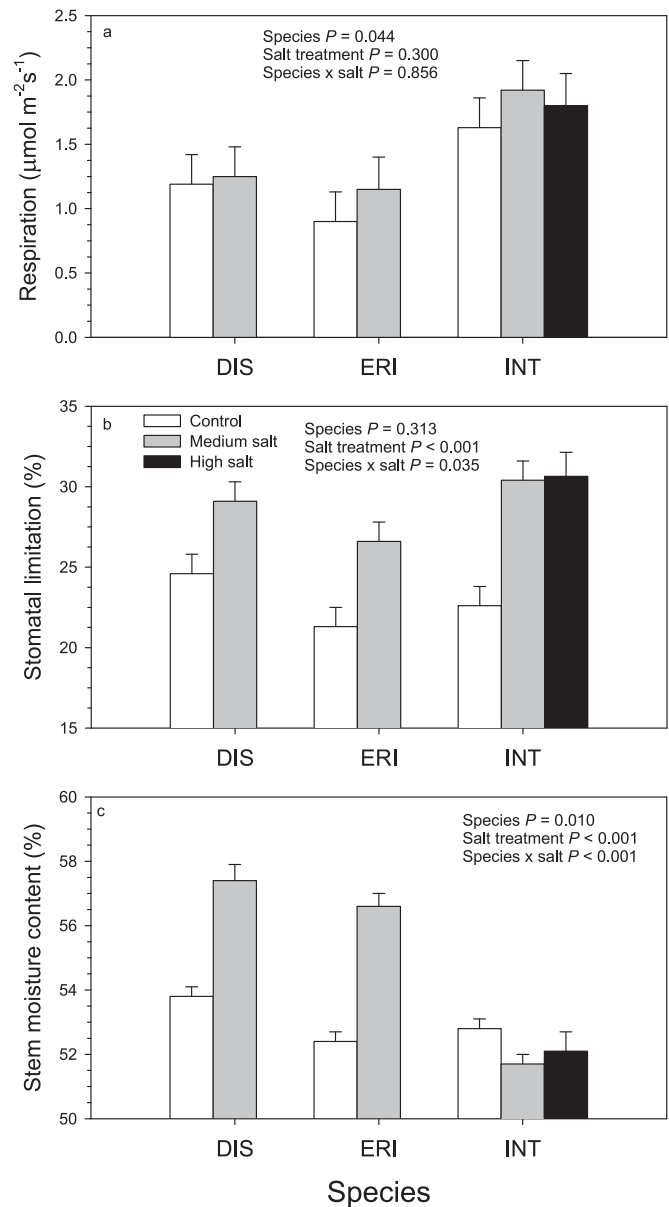

values for INT means. We also present in figures and tables least square mean and SE values from the ANOVA by species and treatments for particular comparisons.

Results

Gas exchange parameters

Species and treatment effects were significant for P_n (Table 2). Species values were 11.0, 12.6, and 18.0 μmol·m^{-2}·s^{-1} for DIS, ERI, and INT, respectively; and treatment values were 12.2, 15.1, and 18.8 μmol·m^{-2}·s^{-1} for CTL, MST, and HST, respectively (Fig. 1*a*). Differences in G_{wv} were significant for species (Table 2), with values of 0.31, 0.41, and 0.40 mmol·m^{-2}·s^{-1} for DIS, ERI, and INT, respectively (Fig. 1*b*). Differences in species and treatment effects were significant for WUE (Table 2). Species values were 41.1, 32.6, and 47.4 for DIS, ERI, and INT, respectively, and treatment values were 35.3, 44.8, and 49.2 for CTL, MST, and HST, respectively

(Fig. 1*c*). Respiration was greatest for INT, followed by DIS and ERI, with values of 1.78, 1.22, and 1.02 μmol·m^{-2}·s^{-1}, respectively (Table 2; Fig. 2*a*). The significant genotype × treatment interaction was the result of INT genotype rank change in HST: INT genotypes 1 and 3 decreased, whereas genotype 2 increased and the other two remained the same. Treatment effect and species × treatment interaction were significant for stomatal limitation (Stomlim) (Table 2). The Stomlim species × treatment interaction was not a result of rank change but of INT not showing a continued increase in the HST (Fig. 2*b*). Overall Stomlim treatment values were 22.8%, 28.7%, and 30.6% for CTL, MST, and HST, respectively.

All sources of variation were significant for stem moisture content (Table 2). The treatment × species interaction was a result of rank change. Stem moisture content was greater in MST than in CTL for DIS and ERI, whereas INT had stable stem moisture content across all three saline treatments, with a value of approximately 52% (Fig. 2*c*). The genotype × treatment interaction reflected DIS and ERI genotypic responses to MST: they ranged from greater than to

equal to stem moisture content relative to CTL, whereas INT genotypes were stable in MST, but there was some variation in HST. The genotype effect was largely due to greater values for INT genotype 2 compared with the other INT genotypes.

Leaf sodium and nutrient parameters

The effects of species and genotype were significant for leaf N and P concentrations (Table 3). Species leaf N values were 2.5%, 2.6%, and 3.3% for DIS, ERI, and INT, respectively (Fig. 3a). Leaf P concentrations were 0.26%, 0.28%, and 0.30% for DIS, ERI, and INT, respectively (Fig. 3b). The significant leaf N genotype effect was largely due to the relative superiority of ERI genotype 3 (GRE-E1) but also the relative inferiority of INT genotype 4 (LON-I4). The significant genotype effect for leaf P was largely due to the relative superiority of ERI genotype 4 (NOR-E10) but also the relative inferiority of INT genotype 4 (LON-I4). Treatment and genotype effects and species × treatment interaction were significant for leaf K^+ concentration (Table 3). The species × treatment interaction was due to magnitude effects in the MST: K^+ leaf concentration for DIS and ERI dramatically increased from CTL to MST, whereas for INT, leaf K in MST remained similar to the CTL treatment. In the HST, INT leaf K^+ modestly increased from MST (Fig. 3c). The genotype effect was due to higher K^+ in INT genotype 2 (LIM-I3) compared with other INT genotypes.

Leaf Ca^{2+} was significant for genotype effect, which was the result of low Ca^{2+} levels for INT genotype 1 (Table 3; Fig. 4a). There were no significant sources of variation for Mg^{2+} (Table 3; Fig. 4b). Leaf Na^+ was significant for species effect, with values of 0.05%, 0.22%, and 0.43% for DIS, ERI, and INT, respectively (Table 3; Fig. 4c). Species and treatment effects were significant for leaf Mn (Table 3). Manganese concentrations were 271, 221, and 99 ppm for DIS, ERI, and INT, respectively, and 147, 232, and 143 ppm for CTL, MST, and HST, respectively (Table 4).

Treatment, species × treatment, and genotype × treatment were significant for Fe^{2+} (Table 3). Species × treatment interaction was due to treatment rank change: under CTL, species were approximately equal; under MST, Fe^{2+} was elevated relative to CTL for DIS and ERI but not for INT (Table 4). Genotype × treatment was due to the variation in the MST response by DIS and ERI. Species, treatment, and genotype effects were significant for leaf S^{2+} concentrations (Table 3). Sulfur concentrations were 0.20%, 0.19%, and 0.31% for DIS, ERI, and INT, respectively, and 0.25%, 0.20%, and 0.30% for CTL, MST, and HST, respectively (Table 4). Genotype effect was largely INT genotype 1 (LAF-I5), which had lowest S compared with the other INT genotypes.

Differences in leaf C were significant for treatment and genotype effects (Table 3). Treatment values were 44.8%, 44.0%, and 43.4% for CTL, MST, and HST, respectively (Table 4). Most of the genotype variation was found within ERI: the superiority of genotype 5 (SAU-E3) versus genotype 2 (FRE-E1), with the lowest value. Leaf C:N ratio was significant for species (Table 3). Species values were 18.2, 18.0, and 13.4 for DIS, ERI, and INT, respectively (Table 4). Leaf Zn and Al had no significant effects, and thus, the results were not presented.

Species and treatment were significant for the K^+:Na^+ ratio (Table 3). Leaf K^+:Na^+ ratio was 99.1%, 68.1%, and 15.5% for DIS, ERI, and INT, respectively (Fig. 5a), and 77.5%, 45.7%, and 11.2% for CTL, MST, and HST, respectively. Treatment and species × treatment were significant for the Ca^{2+}:Na^+ ratio (Table 3). The species × treatment interaction was due to magnitude effects: both DIS and ERI Ca^{2+}:Na^+ ratios declined in MST, whereas the INT Ca^{2+}:Na^+ ratio remained stable across the three saline treatments (Fig. 5b). Treatment and species × treatment were significant for the Mg^{2+}:Na^+ ratio (Table 3). The species × treatment interaction was due to magnitude effects: both DIS and ERI Mg^{2+}:Na^+ ratios declined in MST, whereas the INT Ca^{2+}:Na^+ ratio remained stable across the three saline treatments (Fig. 5c).

Trait relationships

Across species and saline treatments, P_n had a significant positive relationship to total aboveground dry mass ($P = 0.027$, $R^2 = 0.656$; Fig. 6a) and to WUE ($P = 0.040$, $R^2 = 0.604$; Fig. 6b). Across species and saline treatments, P_n had a significant positive relationship to leaf N concentration ($P = 0.011$, $R^2 = 0.758$; Fig. 7a) and to leaf Na^+ concentration ($P < 0.001$, $R^2 = 0.967$; Fig. 7b). Photosynthesis had significant negative relationships to leaf C concentration ($P = 0.006$, $R^2 = 0.780$; Fig. 8a) and to leaf C:N ratio ($P = 0.003$, $R^2 = 0.851$; Fig. 8b).

Discussion

Gas exchange parameters

Depending on the salinity and experimental time, P_n mostly either remains relatively unchanged or responds negatively to salinity (Bohnert and Sheveleva 1998; Fung et al. 1998; Munns and Tester 2008). The three willow species in our study showed a slight P_n stimulation in MST. Some species are able to tolerate high levels of salinity and reach optimal levels of growth under saline conditions (Khan et al. 2000; Parida et al. 2004; Flowers and Colmer 2008). Photosynthetic regulation (P_{nr}) due to environmental change is often observed after some time (weeks to months) (Tjoelker et al. 1998; Centritto and Jarvis 1999; Zhou et al. 2011) and can reflect temporal changes in species C sink demands (Jach and Ceulemans 2000; Rogers and Ellsworth 2002; Ellsworth et al. 2004; Ainsworth et al. 2004; Ainsworth and Long 2005). As salinity increases, energy is required to exclude or compartmentalize Na^+, but eventual negative effects on growth (C sink), caused by osmotic effects outside the roots, are observed after a species reaches specific salinity thresholds (Mirck and Zalesny 2015; Munns and Tester 2008). Thus, changes in C sink strength can result in a feedback effect that can change P_n (more on this later).

We did not find significant saline treatment or saline treatment × species effects for G_{wv}. Salt induces drought-like responses and can often reduce G_{wv} to mitigate water deficiency caused by osmotic stress (Küçükahmetler 2002). However, stomatal limitation values did show saline treatment and saline treatment × species interaction effects. Saline treatments did have greater stomatal limitation, but the saline treatment × species interaction was a magnitude effect, and not a treatment rank change (the species had a consistent treatment effect, but the magnitude among species was different). Interestingly, WUE values had a significant saline treatment and species effects. As expected, WUE increased under increasing salinity, as previously reported (Clough and Sim 1989; Syvertsen and Melgar 2010). *Salix interior* also had the greatest WUE; INT saline tolerance and greater WUE are most probably a reflection of its evolutionary origins in the arid southwestern USA and Mexico (Bebb 1891; Brunsfeld et al. 1991, 1992) where high evapotranspiration may increase soil salinity (Briggs (1996) and references therein) and result in a natural selection for increased saline tolerance.

Leaf sodium and nutrient parameters

Saline tolerance via a root exclusion mechanism minimizes root uptake of antagonistic cations (Na^+) and anions (Cl^-) and is one of three main physiological responses to soil salinity (Munns and Tester 2008; Hangs et al. 2011). For most species, Na^+ appears to reach toxic concentrations before Cl^- does, so most studies have concentrated on Na^+ exclusion and control of Na^+ within plants (Munns and Tester 2008). Interestingly, under CTL conditions, INT had almost 13× the leaf Na^+ concentration than DIS and ERI. However, after this relatively high threshold, it would appear that INT has an active Na^+ root exclusion mechanism, as Na^+ increased by only 17% across each treatment, whereas DIS and ERI leaf Na^+ increased 6× on average from CTL to MST. A general stress response in the plant and animal kingdoms is the accumulation of ions (K^+, Na^+, and Ca^{2+}) and increased amounts of carbohydrates to

Table 3. Leaf sodium, nutrient, and nutrient ratio trait ANOVAs, including source of variation, degrees of freedom (df), mean square values (MS), P values, and coefficient of determination (R^2).

Source of variation	df	Sodium (%) MS	P value	Nitrogen (%) MS	P value	Phosphorus (%) MS	P value	Potassium (%) MS	P value	Calcium (%) MS	P value	Magnesium (ppt) MS	P value	Manganese (ppm) MS	P value	Iron (ppm) MS	P value	Sulfur (%) MS	P value	Carbon (%) MS	P value
Block	1	0.024	0.495	0.096	0.403	0.11×10^{-3}	0.833	0.008	0.835	1.159	0.001	1.563	0.013	0.06	0.965	76.2	0.342	0.0620	**<0.001**	13.7119	**<0.001**
Treatment (trt)	1	0.144	0.102	0.075	0.460	0.13×10^{-3}	0.817	5.020	**<0.001**	0.201	0.128	1.005	0.201	175.02	**0.007**	579.0	**0.003**	0.0231	**<0.001**	5.6966	**0.002**
Species	2	0.247	**0.014**	3.417	**<0.001**	15.28×10^{-3}	**0.016**	0.063	0.721	0.001	0.904	0.174	0.380	342.14	**<0.001**	19.5	0.789	0.0129	**0.013**	2.4344	0.076
Species × trt	3	0.016	0.822	0.343	0.071	4.29×10^{-3}	0.150	0.995	**0.004**	0.241	0.066	0.006	0.972	66.77	0.129	355.4	**0.019**	0.0006	0.788	0.4535	0.605
Genotype (species)	10	0.060	0.339	0.288	**0.046**	8.93×10^{-3}	**0.001**	0.579	**0.008**	0.192	**0.048**	0.416	0.177	11.04	0.946	161.6	0.075	0.0131	**<0.001**	2.8268	**0.002**
Genotype (species) × trt	18	0.031	0.890	0.136	0.471	2.39×10^{-3}	0.474	0.150	0.703	0.060	0.826	0.370	0.229	12.95	0.976	175.1	**0.026**	0.0031	0.325	0.7324	0.479
Error	35	0.051		0.134		2.37×10^{-3}		0.190		0.092		0.228		30.69		81.9		0.0026		0.728	
R^2			0.639		0.791		0.747		0.751		0.655		0.747		0.673		0.724		0.851		0.773

Source of variation	df	C:N ratio MS	P value	K:Na ratio MS	P value	Ca:Na ratio MS	P value	Mg:N ratio MS	P value
Block	1	12.882	0.098	18 015	**0.008**	24 931	**0.006**	566.2	**0.008**
Treatment (trt)	1	9.868	0.146	14 527	**0.017**	57 713	**<0.001**	942.2	**0.001**
Species	2	99.694	**<0.001**	18 991	**0.007**	5586	0.165	129.7	0.182
Species × trt	3	4.026	0.450	2301	0.422	14 939	**0.005**	226.2	**0.038**
Genotype (species)	10	6.586	0.195	2019	0.564	940	0.940	80.6	0.379
Genotype (species) × trt	18	4.330	0.513	1672	0.763	4339	0.152	86.5	0.315
Error	35	4.459		2307		2946		72.5	
R^2			0.785		0.739		0.769		0.740

Note: P values <0.05 are in bold type.

Fig. 3. (*a*) Leaf nitrogen (mean ± SE), (*b*) leaf phosphorus, and (*c*) leaf potassium concentrations by willow species under different salt treatments. Species: DIS, *Salix discolor*; ERI, *S. eriocephala*; INT, *S. interior*.
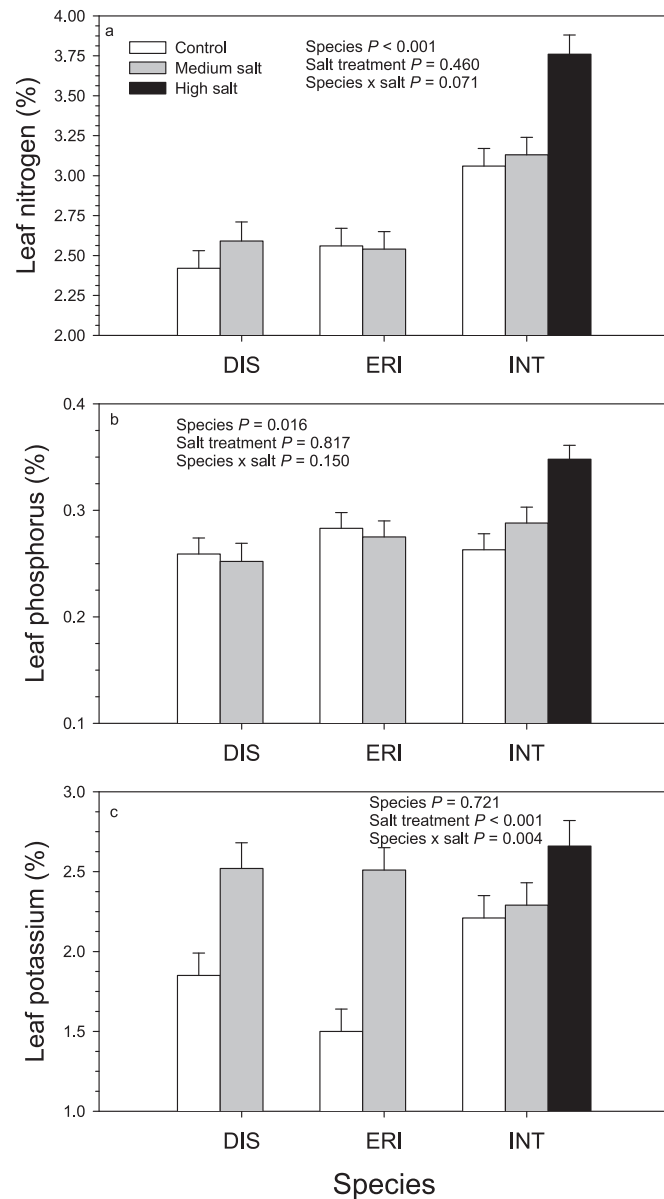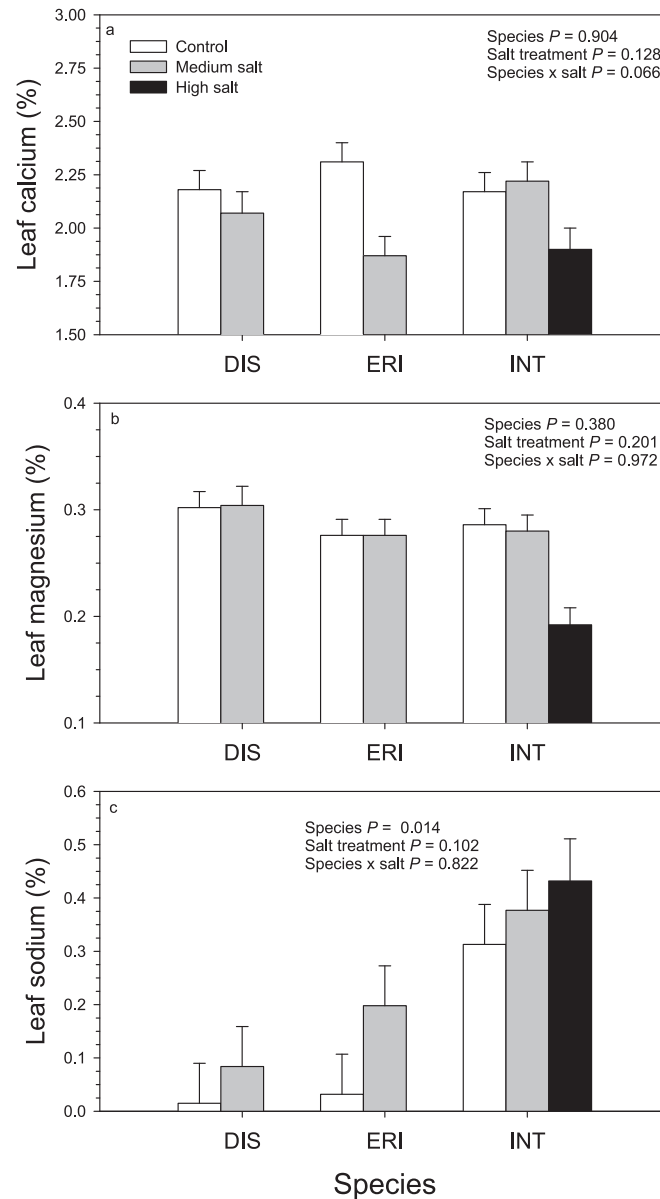
Fig. 4. (*a*) Leaf calcium (mean ± SE), (*b*) leaf magnesium, and (*c*) leaf sodium concentrations by willow species under different salt treatments. Species: DIS, *Salix discolor*; ERI, *S. eriocephala*; INT, *S. interior*.

increase cellular osmotic potential (Bohnert and Sheveleva 1998). However, under saline stress, there is often a decrease in K+, Ca2+, and Mg2+ in plant tissues, depending on the species, due to a competitive interaction (or exclusion) with Na+ (Valdez-Aguilar et al. 2009; Dong et al. 2013). All three willows showed species-specific cation responses to MST: for DIS, K+ increased and Ca2+ and Mg2+ remained the same as for CTL; for ERI, K+ increased, Ca2+ decreased, and Mg2+ remained the same; and for INT, K+ increased only in HST and Ca2+ and Mg2+ decreased but only in HST.

Two other main physiological responses to saline include (*i*) transport and storage of Na+ in leaf and stem tissues, which can also contribute to (*ii*) osmotic and photosynthetic adjustments (Flowers and Colmer 2008; Munns and Tester 2008). Plant mortality is thought to occur when leaf mortality becomes faster than new leaf production, brought about by an increased influence of salt ions, which upon reaching a "maximum" concentration results in mortality (Munns and Termaat 1986; Munns et al. 2006). Some species do not show much increase in salt concentration

over time and thus can avoid leaf mortality, whereas other species exclude or store salt in vacuoles, thereby controlling uptake and compartmentalization of ions such as Na+, Cl−, Fe2+, and S2− (Flowers and Colmer 2008; Zhang et al. 2015). Among these ions, Na+ is considered the main ion negatively affecting plant growth. In our study, Na+ and also K+ concentrations in plants increased with increasing salinity, and both elements help with osmotic adjustment during saline stress. However, Na+ must be effectively partitioned and confined to cell vacuoles. Exactly how plants direct Na+ to the vacuole is unclear (Flowers and Colmer 2008).

It is clear that DIS and ERI are negatively affected and are sensitive to salinity, which they only just tolerated in MST. The results also show that INT is more saline-tolerant. Again, the ability to maintain high K+:Na+, Ca2+:Na+, and Mg2+:Na+ ratios under saline conditions is a strong indicator of saline tolerance (Bohnert and Sheveleva 1998; Cassaniti et al. 2013). Interestingly, DIS and ERI initially had greater cation:Na+ ratios than INT; however, INT cation:Na+ ratios were stable across the three saline treatments,

Table 4. Nutrient, element, and C:N ratio (mean ± SE) by willow species and salt treatments.

Nutrient	Salt treatment	*Salix discolor*	*Salix eriocephala*	*Salix interior*
Iron (ppm)	Control	46.5±3.5	50.3±3.5	52.9±3.5
	Medium	65.0±4.0	56.0±3.5	48.3±3.5
	High			51.8±4.7
Manganese (ppm)	Control	247.4±36.9	137.2±36.9	57.5±36.9
	Medium	294.4±42.0	304.8±35.7	97.2±36.9
	High			143.0±36.9
Sulfur (%)	Control	0.227±0.014	0.221±0.014	0.321±0.014
	Medium	0.182±0.016	0.156±0.014	0.276±0.014
	High			0.345±0.021
Carbon (%)	Control	45.1±0.25	45.3±0.25	44.1±0.25
	Medium	44.0±0.25	44.4±0.25	43.6±0.25
	High			43.4±0.30
Carbon:nitrogen ratio	Control	19.1±0.7	18.1±0.7	14.5±0.7
	Medium	17.3±0.7	17.8±0.7	14.1±0.7
	High			11.7±0.7

Fig. 5. (*a*) Leaf K:Na ratio (mean ± SE), (*b*) leaf Ca:Na ratio, and (*c*) leaf Mg:Na ratio by willow species under different salt treatments. Species: DIS, *Salix discolor*; ERI, *S. eriocephala*; INT, *S. interior*.

whereas cation:Na^+ ratios in DIS and ERI dropped precipitously from CTL to MST. Several K^+ channels and transporters across the cell membranes have been discovered (see review by Maathuis et al. 1997). Differential gene expression in response to salinity involved photosynthesis-related genes, which were upregulated in male *Populus yunnanensis* trees (Jiang et al. 2012). High plant Ca^{2+} and Mg^{2+} concentrations have been reported to mitigate saline stress (Hangs et al. 2011); however, we did not see any species or saline effects for leaf Ca^{2+} or Mg^{2+}.

Saline-tolerance mechanisms require a diversion of energy from plant growth to increased respiration for salt pumping or storage, generally resulting in reduced productivity. Alternatively, an increase in energy supply may be stimulated (Bohnert and Sheveleva 1998; Cassaniti et al. 2013). *Salix discolor* and ERI can cope with the MST only by reallocating energy from productivity to deal with increased NaCl. The three willow species upregulated P_n and biochemical efficiencies V_{cmax} and J_{max}, allowing plants to meet new sink demands (Major et al. 2017); however, INT was the only species that was also able to translate this into sustained or increased total aboveground biomass production. Although leaf respiration did not change with saline treatment, consistent with findings by Papp et al. (1983) in a review of respiratory responses to salinity, it may be more complex, as 37% of studies reviewed reported a respiratory increase, 34% reported a decrease, and 29% saw no consistent changes (Jacoby et al. 2011). *Salix interior* had slightly greater leaf respiration than DIS and ERI, and this may reflect the significantly greater energy expended in translocation or exclusion of salt. Saline tolerance is a multigene trait caused by many morphological, physiological, and biochemical adaptations (Munns and Tester 2008; Polle and Chen 2015). The suggestion of Fung et al. (1998) of selection and breeding for species and genotypes that exhibit greater photosynthesis is supported by our observations of both species and genotypic variations in saline tolerance.

One of the three mechanisms by which plants can physiologically adjust to saline environments is to accumulate organic and inorganic solutes to regulate osmotic potential. An increase in salinity generally lowers leaf osmotic potential (Cassaniti et al. 2013) and thus helps generate turgor to overcome cell wall resistance to growth (Lockhart 1965; Johnsen and Major 1999; Munns and Tester 2008). However, it would appear that DIS and ERI are not able to effectively compartmentalize the Na^+. It is interesting that the two saline-intolerant species, DIS and ERI, had greater stem moisture content in MST compared with CTL, whereas INT genotypes were stable across the three treatments. This suggests that DIS and ERI may be absorbing water to neutralize the effects of salinity, resulting in loosely held water (Parida et al. 2004; Munns and Tester 2008).
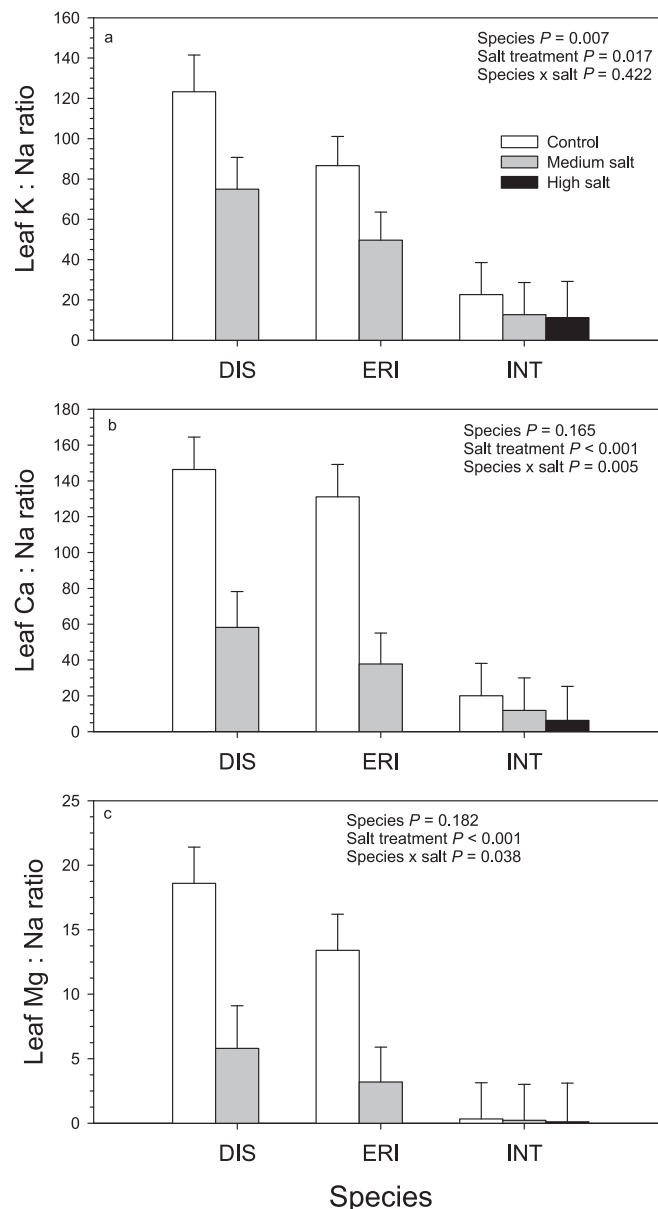
In an assessment of foliage nutrients on acidic (pH = 3.6) and neutral (pH = 6.8) mine-site soils, the mean leaf N concentrations in DIS and ERI were 1.5% and 1.9%, respectively, with slightly lower values on the acidic site (A. Mosseler and J.E. Major, unpublished). These were lower than N values found for DIS and ERI in this experiment (2.5% and 2.6%, respectively). Foliage K under saline treatment was 2.5%, which was 2.5× that found on the acidic and neutral mine site, which had 1.0% K.

There was no significant genotype or genotype × treatment interaction for any gas exchange trait. There was no significant genotype × treatment interaction for any of the nutrient elements or parameters. There were six significant genotype effects (N, P, K^+, Ca^{2+}, C, and S^{2-}); however, there were no consistent trends or genotypes that stood out for these parameters. In contrast, there were significant genotype biomass yield and survival traits for all but one trait (Major et al. 2017). This is most probably due to the fact that there were more replicates for biomass than gas exchange and leaf nutrient sampling, which allowed for better

Fig. 6. Relationship between net photosynthesis (mean ± SE) and (*a*) total aboveground (AG) biomass and (*b*) water-use efficiency by willow species under different salt treatments. Species: DIS, *Salix discolor*; ERI, *S. eriocephala*; INT, *S. interior*. Salt treatments: C, control; M, medium; H, high salt treatment.
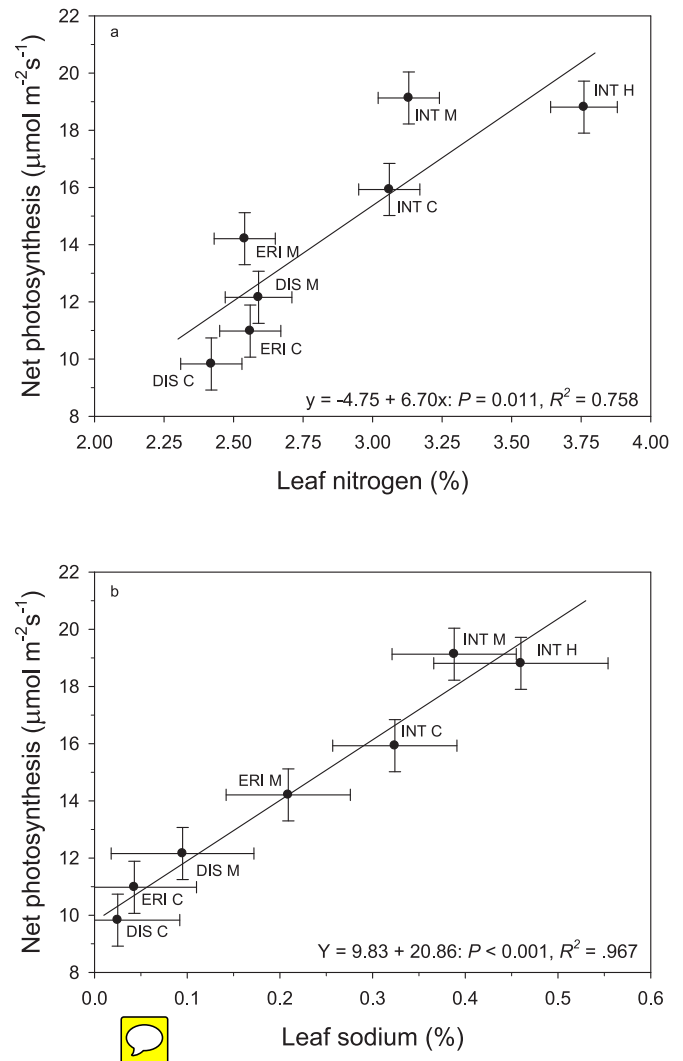
Fig. 7. Relationship between net photosynthesis (mean ± SE) and (*a*) leaf nitrogen and (*b*) sodium concentration by willow species under different salt treatments. Species: DIS, *Salix discolor*; ERI, *S. eriocephala*; INT, *S. interior*. Salt treatments: C, control; M, medium; H, high salt treatment.
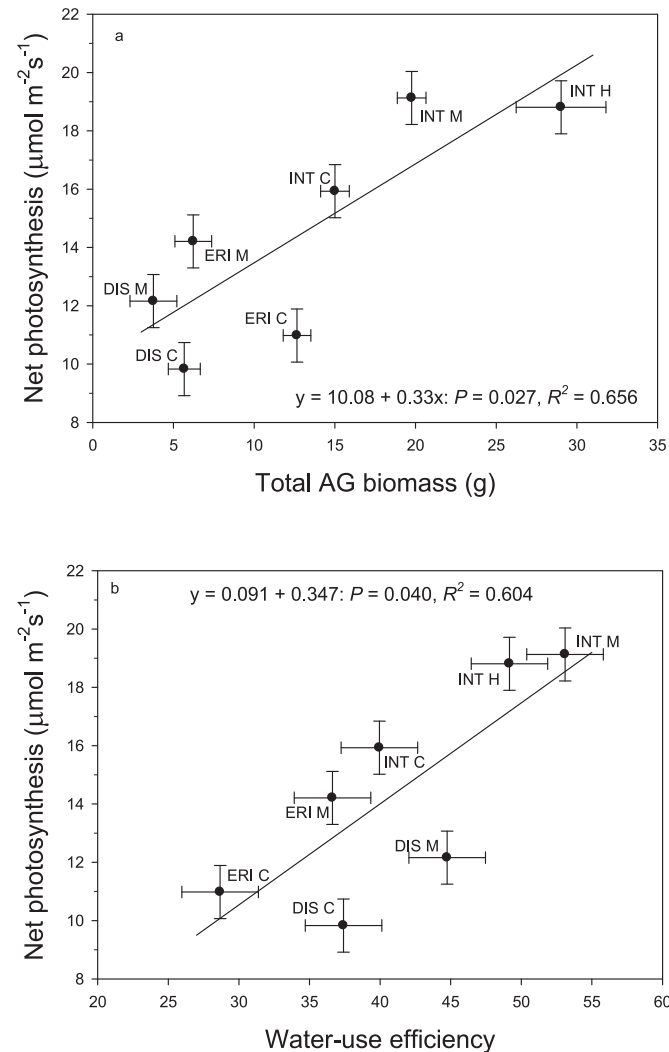
resolution. However, it may be that the gas exchange and leaf nutrient traits, although significant by species and treatments effects, are by nature genotypically more variable than biomass yield traits.

Relationships to other traits

Why is P_n highest for INT in the HST and lowest for DIS in the CTL? The sink regulation of P_n theory suggests examining the total dry mass demand (Paul and Foyer 2001; Ainsworth et al. 2004). This theory suggests that greater sink demand (growth) stimulates greater P_n. Across species and saline treatments, P_n had a significant positive relationship to total aboveground dry mass ($P = 0.027$). Another causative factor is that saline tolerance requires energy (soluble carbohydrates) to cope in stressful environments (Jacoby et al. 2011). Again, P_n increased in all three species in the MST, a direct response to saline stress; however, INT was the only species to increase biomass in each progressive saline treatment. The increase in biomass was not a result of increased number of stems or longer stem lengths but of an increase in basal diameter (Major et al. 2017). It is not uncommon for saline-tolerant plants to increase stem diameter or mass in response to saline stress (Khan et al. 2000; Parida et al. 2004; Flowers and Colmer 2008). Pho-
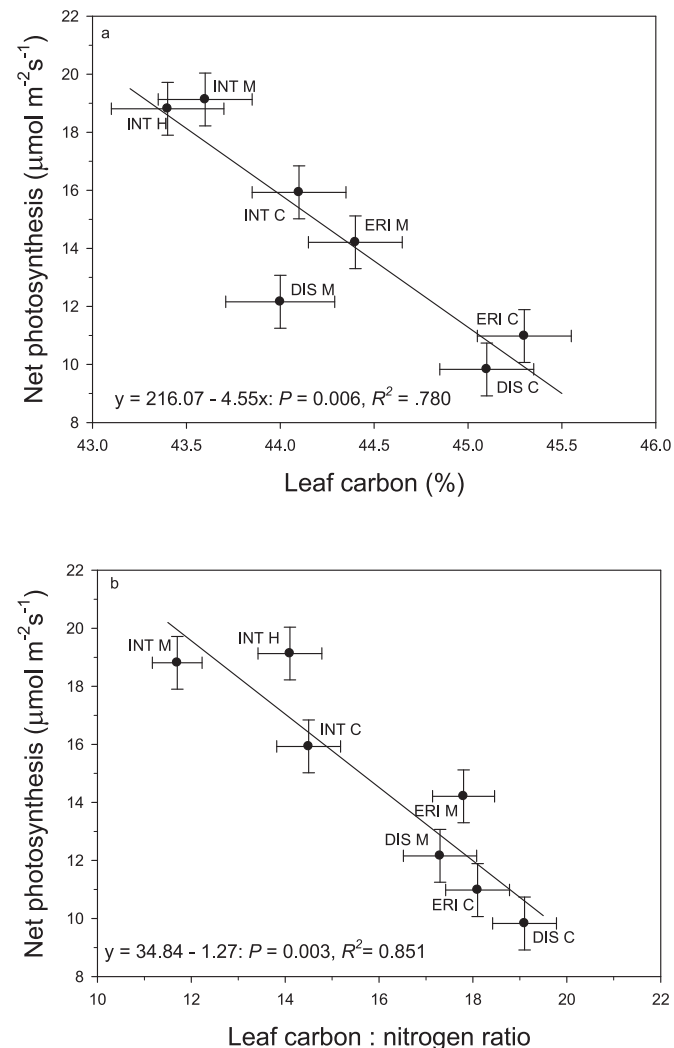
tosynthetic traits have been correlated with productivity (Major and Johnsen 1996; Reich et al. 1998; Tjoelker et al. 1998; Johnsen et al. 1999), but it is clear that the correlations among photosynthesis, growth rate, and chlorophyll content in forest trees can be complicated and depend on many factors such as time scale, environmental conditions, species, and age (Pereira 1995; Reich et al. 1998). It does appear that sink (growth) demand driven feedback is a consistent driver of P_n among willow species across saline treatments.

Water-use efficiency is a function of P_n and G_{wv}. Changes in either P_n or G_{wv} can affect the WUE. In our study, it was an increase in P_n while there were no changes to G_{wv} that drove the increase in WUE in saline treatments. This was also reflected by the positive P_n relationship to WUE. The P_n relationship to leaf N supports the greater biochemical capacity, as N is an essential element for amino-acid biosynthesis occurring in the chloroplasts (Taiz and Zeiger 1997; Reich et al. 2014). Interestingly, there was a positive relationship between P_n and leaf Na+, which is partially due to the increased energy requirement for excluding or storing Na+. Under stress (i.e., salinity or drought stress), plants alter biochemical mechanisms, including C and N storage and allocation (Bohnert and Sheveleva 1998). Total leaf carbon consistently decreased under saline treatments across all three willow species. P_n

Fig. 8. Relationship between net photosynthesis (mean ± SE) and (*a*) leaf carbon concentration and (*b*) carbon to nitrogen ratio by willow species under different salt treatments. Species: DIS, *Salix discolor*; ERI, *S. eriocephala*; INT, *S. interior*. Salt treatments: C, control; M, medium; H, high salt treatment.

the arid regions of the southwestern USA, where species of *Salix* section Longifoliae, which includes INT, may have their evolutionary origins.

Acknowledgements

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had significant negative relationships to leaf C ($P = 0.008$, $R^2 = 0.780$) and C:N ratio ($P = 0.003$, $R^2 = 0.851$), the latter reflecting the decline in C and greater N concentration for INT in HST.

Summary

Overall, INT had the greatest P_n and WUE. From CTL to MST, P_n increased for all species and remained at the same level for INT in HST. It would appear that INT displays active Na^+ exclusion at the higher Na^+ concentration as Na^+ only increased on average by 17% across each saline treatment, whereas DIS and ERI leaf Na^+ increased on average 6.0× from CTL to MST. *Salix discolor* and ERI had greater initial cation:Na^+ ratios than INT; however, INT ratios were stable across the three saline treatments, whereas the DIS and ERI ratios showed a precipitous drop from CTL to MST. Across species and saline treatments, P_n had significant positive relationships to total aboveground dry mass, leaf N, and leaf Na^+. Results indicate that INT is a comparatively saline-tolerant willow species that is able to maintain greater P_n, WUE, major nutrient acquisition, and ambient Na^+ storage and exclusion compared with DIS and ERI. This may be the result of adaptation to increased natural selection pressures from increased salinity levels faced by INT in

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