



# Article Assimilation Efficiencies and Gas Exchange Responses of Four Salix Species in Elevated CO<sub>2</sub> under Soil Moisture Stress and Fertilization Treatments

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Abstract: Assimilation to the internal  $CO_2$  (ACi) response curve and gas exchange parameters were quantified for four North American willows ((Salix cordata (COR), S. discolor (DIS), S. eriocephala (ERI), and S. interior (INT)) grown in a  $2 \times 2$  factorial of atmospheric CO<sub>2</sub> and soil moisture treatments to see how they would respond to climate change factors. After the first year of greenhouse growth under said treatments, we saw no difference in the aboveground stem biomass between CO<sub>2</sub> treatments. Thus, in the second year, a second experiment on a subset of well-watered, coppiced willows was conducted in a 2  $\times$  2 factorial of atmospheric CO<sub>2</sub> and soil fertilization (FERT) treatments. In both experiments, the maximum rate of carboxylation ( $V_{cmax}$ ) significantly declined for all four species in response to elevated  $CO_2$  (eCO<sub>2</sub>). In response to a drought treatment (DRT),  $V_{cmax}$  declined, except for INT, which increased V<sub>cmax</sub>. In both experiments, INT had the greatest V<sub>cmax</sub>, maximum rate of electron transport  $(J_{max})$ , and triose phosphate utilization, followed by COR and ERI, with DIS having the lowest values. FERT resulted in a strong increase in assimilation (A) and stomatal conductance  $(G_{wv})$  by 92 and 119%, respectively.  $G_{wv}$  is the primary driver and A is a minor driver of water use efficiency (WUE) under DRT. FERT mitigated the V<sub>cmax</sub> and A downregulation in eCO<sub>2</sub>, but eCO<sub>2</sub> did not mitigate the DRT downregulation effect. Differences between INT and the other three willows in a number of adaptive traits and responses related to drought may reflect the evolutionary origins of INT and the taxonomic group Longifoliae in the arid southwest USA and Mexico.

**Keywords:** assimilation to internal CO<sub>2</sub> curves; elevated CO<sub>2</sub>; gas exchange; net photosynthesis; *Salix* species; soil fertilization; soil moisture stress; stomatal conductance; water use efficiency

## 1. Introduction

Ecological adaptations related to carbon assimilation are important to plant fitness because they affect growth and survival in different environments and this has implications for interspecific competition. Assimilation traits vary widely among species, environments, and plant age [1,2]. Assimilation downregulation ( $A_{dr}$ ) of the photosynthetic apparatus (e.g., chlorophyll and biochemical efficiency traits), also known as biochemical downregulation, often occurs in response to elevated CO<sub>2</sub> (eCO<sub>2</sub>) and results in the reduction in maximum carboxylation efficiency ( $V_{cmax}$ ) and maximum electron transport ( $J_{max}$ ) among other related traits [3–6]. Studies have shown that photosynthetic apparatus downregulation generally ranges from no downregulation to complete downregulation (when assimilation (A) is the same under eCO<sub>2</sub> as it is under ambient levels), depending on species and study [2,7].

With climate warming, frequency and severity of drought are predicted to increase [8]. Water is a predominant factor in determining the geographic and landscape distribution of vegetation, and drought is an important factor in reducing plant growth and increasing mortality [9]. Physiologically, water stress and heat reduces plant and leaf morphology, water potential, photosynthesis, biochemical responses, and can increase photo-oxidative



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**Copyright:** © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). stress and leaf abscission, [10–12]. Tree physiological responses to water availability vary among and within species [10,12,13].

*Salix cordata* Michx. (COR), *S. discolor* Muhl. (DIS), *S. eriocephala* Michx. (ERI), and *S. interior* Rowlee (INT) are native to eastern and central North America and are promising as fast-growing sources of woody biomass production [14]. *Salix cordata*, also known as sand dune willow, is commonly associated with sand dune landscapes of the Great Lakes—St. Lawrence River of northeastern North America. *Salix interior* is also widespread in western North America, ranging from Mexico to Alaska and has been observed to be saline tolerant in greenhouse experiments [15,16]. *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.

The predicted doubling of  $CO_2$  this century and increasing drought with anticipated global warming may affect comparative physiological processes among species. There are few climate change physiological response studies for North American willow species. We could only find one study for willow response to eCO<sub>2</sub> and it had a water stress treatment, using a single clone of *Salix sagitta* [17]. For willow responses to drought, we found three studies: [18] using S. purpurea L.; [19] with S. nigra Marshall, and [20] with a larger group of willows that included S. candida Fluegge ex Willd., S. pedicellaris Pursh, S. pyrifolila Andersson, S. bebbiana Sarg., S. discolor, and S. petiolaris Sm. Our goal was to examine and compare assimilation efficiencies and gas exchange (GE) traits for four North American willow species under the interactive effect of atmospheric  $CO_2$ , soil moisture, and soil fertilization (FERT) treatments. We hypothesized that eCO2, soil moisture stress, and FERT responses in willows would differ among species. To test the hypothesis, we (1) quantified the four willow species' genetic variation in assimilation to internal  $CO_2$  (ACi) response curves and *GE* parameters, in response to  $eCO_2$ , soil moisture stress, and FERT treatments, and (2) tested the three treatment effects on the species' physiological interrelationships using covariate analysis.

#### 2. Materials and Methods

This greenhouse study grew plants from dormant willow cuttings under  $CO_2$  and soil moisture treatments over two growing seasons (2017 and 2018), with the above-ground biomass harvested after the first year, using the coppice regrowth during the second year of treatments. In the second year, a sub-set of well-watered coppice plants were given different levels of fertilizer.

#### 2.1. Plant Material, Growing Conditions, and Treatment Delivery

Sixteen genotypes (clones), four clones for each of the four willow species, COR, DIS, ERI, and INT were used for this study (Table 1). Dormant stem cuttings approximately 20 cm long were collected either in the field in April 2017 and kept frozen (-8 °C), or in early May 2017 and kept cool (4 °C), in sealed plastic bags until 7 June 2017 when all the stored stem cuttings were moved to a common 4 °C cooler. Cuttings were placed in water at room temperature for two days, prior to potting on 13 June 2017.

Cuttings were rooted and grown individually in fabric root-control bags 30 cm in diameter and 23 cm deep (Smart Pot PRO 5 Gallon, High Caliper Growing Systems, Oklahoma City, OK, USA) filled with sandy soil. Four bulk soil samples that were sent for texture analysis to the University of New Brunswick nutrient analyses laboratory, showed sand, silt, and clay portions, of  $87.5 \pm 0.7$ ,  $7.8 \pm 0.4$ ,  $4.8 \pm 0.3$  (mean + S.E.), respectively. Root bags were arranged on a sand-covered gravel surface, inside eight custom-made chambers, each 2.3 m wide  $\times 4.25$  m long  $\times 2.1$  m high, covered in UVA-clear 4 mil polyethylene film, with outside air delivered and circulated by positive pressure fans. Each chamber contained four randomly distributed replicates (ramets) of each genotype—thus, 64 plants per chamber (4 ramets  $\times 16$  clones),  $\times$  eight chambers (a total of 32 ramets per genotype). The eight chambers were housed inside two separate greenhouse compartments

(two blocks of four chambers) at the Canadian Forest Service–Atlantic Forestry Centre (CFS–AFC) in Fredericton, NB, Canada (45°52′ N, 66°31′ W).

**Table 1.** Species and clones of *Salix* used in 2017  $CO_2 \times soil$  moisture and 2018  $CO_2 \times fertilization experiments.$ 

<u>Grandina</u>	<i>a</i> 1 <i>v</i> 5	P	T / T	Measured		
Species	Clone ID	Provenance	Lat-Long -	2017	2018	
S. cordata	BIG-C4m	Big Sandy Bay, ON	44°06′ N–77°43′ W	Х		
	BIG-C5f	Big Sandy Bay, ON	44°06′ N–77°43′ W			
	NOR-C4f	North Beach Park, ON	43°56′ N–77°31′ W	Х	Х	
	OUT-C1m	Outlet Beach, ON	43°53′ N-77°13′ W		Х	
S. discolor	HAW-D4m	Hawkesbury, ON	45°36′ N–74°36′ W	Х		
	HAW-D5f	Hawkesbury, ON	45°36' N–74°36' W		Х	
	MON-D1f	Montmagny, QC	46°58′ N–70°33′ W	Х	Х	
	RIC-D2m	Richmond Fen, On	45°07′ N-75°42′ W			
S. eriocephala	ALL-E2m	Allumette Island, QC	45°54′ N-77°06′ W	Х		
	FRE-E1f	Fredericton, NB	45°57′ N–66°38′ W		Х	
	GRE-E1f	Green River, NB	47°34′ N–68°19′ W	Х	Х	
	WAI-E1m	Wainfleet, ON	42°55′ N–79°20′ W			
S. interior	LAF-I2f	Ottawa, ON	45°25′ N–75°41′ W	Х	Х	
	LAF-I12m	Ottawa, ON	45°25′ N-75°41′ W			
	WAI-I1m	Wainfleet, ON	42°55′ N–79°20′ W	Х		
	WAI-I2f	Wainfleet, ON	42°55′ N–79°20′ W		Х	

Plants were grown under natural daylength with light levels inside the chambers approximately 65% of outside levels, determined according to [21]. Temperatures inside the chambers were controlled by venting the greenhouse chambers, and by individual air conditioning units within each chamber. Water was delivered to each rooting bag via individual spigots in a dripper system. All root bags were kept well-watered for the first two weeks prior to starting the treatments, to allow for rooting of the dormant stem cuttings. A custom-made logging and control system continuously monitored sensors inside each chamber for air temperature and humidity (model HMP 155, Vaisala, Vantaa, Finland, and model AM2302, Adafruit Industries, New York, NY, USA), soil moisture (volumetric water content VWC), temperature (model 5TM, Meter Group, Pullman, WA, USA), and CO<sub>2</sub> concentration sampled at plant height, mid-chamber (Model 840A, LI-COR Inc., Lincoln, NE, USA).

In each greenhouse, each chamber was randomly assigned to one of four treatment types (2  $\times$  2 factorial of two CO<sub>2</sub> treatments and two irrigation levels). CO<sub>2</sub> treatments were ambient (aCO<sub>2</sub>—no CO<sub>2</sub> added, approximately 400 ppm) and elevated (eCO<sub>2</sub>—target of 800 ppm), with eCO<sub>2</sub> treatment maintained by the system opening or closing solenoid valves to control delivery of CO<sub>2</sub> into the outside air stream. Irrigation treatments were well-watered (WW, volumetric water content target of 20–25%) and drought (DRT, VWC 10–15%). Irrigation levels were controlled by manually powering valves to deliver water as needed to maintain treatment VWC target levels. CO<sub>2</sub> treatments were started on June 28, 15 days after cuttings were potted. By this time, all cuttings were leafing out. Drought treatment was allowed to progress gradually, beginning June 28.

Fertilizer (Plant Prod "Forestry Special" 20:8:20 delivered at 100 ppm N) was supplied to all plants via the dripper system on only two dates in 2017: 5 July (30 min = 1 L) and 24 July (15 min = 500 mL). Treatments ran until 31 October 2017, when above-ground

portions of all plants were harvested. For the winter of 2017–2018,  $CO_2$  enhancement was stopped, and soil in all bags was kept well watered. Temperatures were kept at a minimum, just above 0 °C, to avoid freezing greenhouse infrastructure, and blackout curtains were used to prevent solar heating.

#### 2.2. Assimilation Efficiencies and Gas Exchange Experiment: 2017

From 12–26 September 2017 (days 76–90 of treatment), GE measurements were performed on two clones per species (Table 1. Species and clones of Salix used in  $2017 \text{ CO}_2 \times \text{soil moisture and } 2018 \text{ CO}_2 \times \text{fertilization experiments}$ ) in situ using a LI-COR 6400XT portable GE system with a CO<sub>2</sub> mixer and RGB (Red Green Blue) LED light source on a 2  $\times$  3 cm leaf cuvette (LI-COR, Lincoln, Nebraska, USA). On each measurement day, approximately eight assimilation to internal  $CO_2(AC_i)$  response curves were completed by sampling one ramet of one genotype from each of two species from one replicate chamber per treatment. This pattern was repeated for the other two species on the following day. Species pairs were changed with successive paired dates. Over four such pairs of days, this resulted in all 8 genotypes being measured in two replicate chambers for each of the four treatment combinations (64 curves). Instrument settings were set for leaf temperatures of 25–28 °C, air flow of 250–400 umol  $\cdot$ s<sup>-1</sup>, and a saturating photosynthetically active radiation (PAR) level of 1000  $\text{umol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Relative humidity (RH) averaged 50%, and was adjusted to maintain a vapour pressure deficit (VPD) of approximately 1.75 kPa in the chamber. Gas exchange was measured 13 times at 11 different  $CO_2$  levels in the following sequence: 400, 330, 260, 190, 120, 50, 400, 400, 600, 800, 1200, 1700, and 2200 ppm. Upon initially securing the leaf in the chamber, no measurements were taken for 15–30 min, while the leaf equilibrated to the conditions in the chamber. Equilibration was judged by stability in the rate of net photosynthesis. Each subsequent measurement had a settling time of at least 2 min after the new  $CO_2$  was achieved. Two consecutive measurements were performed at 400 ppm following the low reading at 50 ppm, to allow for the slower acclimation following an increase in [CO<sub>2</sub>]. Note that for ACi parameter determinations, values from all three measurements at 400 ppm were averaged to represent that  $CO_2$  concentration level.

The *ACi* response curves were fitted using commercially available software (Photosyn Assistant; Dundee Scientific, Dundee, Scotland, UK). This program uses algorithms based on the biochemical model of leaf photosynthesis developed by [22], and modified by [23]. Using this model,  $CO_2$  assimilation in the leaf can be mathematically described by saturation kinetics, which account for the change in assimilation (*A*) rate at increasing internal  $CO_2$  (*Ci*). Through an iteration procedure, the Photosyn Assistant software provides estimates for the maximum rate of carboxylation ( $V_{cmax}$ ), light saturation rate, maximum rate of electron transport ( $J_{max}$ ), and phosphorus (P) limited rate of triose phosphate utilization (*TPU*). The model calculations are based on the assumption that one of these parameters is at a maximum and limits *A*. In the program, a least-squares fit is used to estimate the  $CO_2$  compensation point.

In addition, *GE* parameters were examined at a common 600 ppm CO<sub>2</sub>, the near mid-point of the two CO<sub>2</sub> treatments, and thus *A* at 600 ppm CO<sub>2</sub> is abbreviated as  $A_{600}$ . Other *GE* parameters examined were stomatal conductance at 600 ppm CO<sub>2</sub> ( $G_{wv600}$ ) and water use efficiency at 600 ppm CO<sub>2</sub> (*WUE*<sub>600</sub>), determined by dividing  $A_{600}$  by  $G_{wv600}$ .

#### 2.3. $CO_2 \times$ Fertilizer Experiment: 2018

On 23 April 2018, the blackout curtains were opened and  $CO_2$  treatments began with the same methods and targets as in 2017. On the same day, differential watering regimes began to re-establish the two irrigation treatments. Irrigation and  $CO_2$  treatments were continued until 10 September 2018, when all plants were harvested.

On 19 July 2018, sixteen plants from each of the four well-watered chambers were selected; two ramets from each of two clones (genotypes) per species. Paired ramets in each chamber were selected to be similar in size. These 64 plants had their root bags briefly lifted and replaced inside plastic bags, to isolate their root systems and contain water delivered

to the root bags. In each chamber, one ramet of each genotype was randomly assigned to the fertilized treatment (FERT), the other to non-fertilized (NONFERT). Watering spigots were removed from all FERT bags. For the remainder of the experiment, NONFERT plants received the same watering regime as all other plants via spigots, while FERT plants were hand-watered with an equal volume of water (typically 1 L two times per week) containing fertilizer (Plant Prod "Forestry Special" 20:8:20 delivered at 100 ppm N). When water was seen to accumulate in the NONFERT plastic bags, they were all pierced at the base to allow water to drain. Water accumulation did not occur with the FERT plants.

*ACi* curves were measured on 10 days between 7 and 20 August 2018 (days 18–31 of fertilizer treatment) on all 64 plants in the fertilizer experiment. Species, clones, and treatments were sampled randomly on each day. Methods were as in 2017, except PAR was set to 500 umol·m<sup>-2</sup>·s<sup>-1</sup> and leaf temperature ranged from 24.5 to 30 °C. Although light levels were different between years, both were above the light saturation point for individual leaves. Eight bulk soil samples (one bulked from FERT and one from NONFERT pots for each chamber) were collected in early September 2018 from NONFERT and FERT treatments and sent to the University of New Brunswick nutrient laboratory for analyses. The soil analysis procedure is the same as used in [24].

#### 2.4. Statistical Analysis

This study was established as a completely randomized block experimental design. Willow species,  $CO_2$  level, and soil water treatments were all considered as fixed effects. The data were subjected to analyses of variance (ANOVA) using the following ANOVA model:

$$Y_{iiklm} = \mu + B_i + S_j + C_k + W_l + SC_{ik} + SW_{il} + CW_{kl} + SCW_{ikl} + e_{iiklm}$$
(1)

where Y<sub>ijklm</sub> is the dependent seedling trait of the i<sup>th</sup> greenhouse chamber, of the j<sup>th</sup> willow species, of the  $k^{th}$  CO<sub>2</sub> treatment, of the  $l^{th}$  water treatment, of the  $m^{th}$  stem cutting, and  $\mu$ is the overall mean.  $B_i$  is the effect of the i<sup>th</sup> greenhouse chamber (i = 1, 2), S<sub>i</sub> is the effect of the j<sup>th</sup> species (j = 1, 2, 3, 4),  $C_k$  is the effect of the k<sup>th</sup> CO<sub>2</sub> treatment (k = 1, 2), W<sub>1</sub> is the effect of the  $l^{th}$  water treatment (l = 1, 2), SC<sub>jk</sub> is the interaction effect of the  $j^{th}$  willow species and the k<sup>th</sup> CO<sub>2</sub> treatment, SW<sub>il</sub> is the interaction effect of the j<sup>th</sup> willow species and  $l^{th}$  water treatment,  $CW_{kl}$  is the interaction effect of the  $k^{th}$  CO<sub>2</sub> treatment and the  $l^{th}$ water treatment, SCW<sub>ikl</sub> is the interaction effect of the j<sup>th</sup> willow species, k<sup>th</sup> CO<sub>2</sub> treatment and I<sup>th</sup> water treatment, and eiiklm 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 normality and equality of variance assumptions. The general linear model from Systat (Chicago, IL, USA) was used for analysis. Willow species source of variation, if significant was post-hoc tested using the Tukey mean separation test, was (p = 0.05). The variance component analysis in the ANOVA tables was carried out using the sum of squares as outlined by [25]. In the second experiment, the same ANOVA model was used, but water (W) was replaced with FERT (F).

Covariate analysis was used to evaluate the relationships among mean willow ACi traits and among GE traits and to test treatment effects. In these analyses, the dependent trait (i.e.,  $A_{600}$ ) is examined in relation to three sources of variation were studied: (1) covariate (i.e.,  $G_{wv600}$ ), (2) independent effect (i.e., CO<sub>2</sub> treatment), and (3) independent effect × covariate (i.e., CO<sub>2</sub> treatment ×  $G_{wv600}$ ). The analyses were done based on the following model:

$$Y_{ij} = B_0 + B_{0i} + B_1 X_{ij} + B_{1i} X_{ij} + e_{ij}$$
(2)

where  $Y_{ij}$  is the dependent trait of the i<sup>th</sup> species of the j<sup>th</sup> genus treatment.  $B_0$  and  $B_1$  are average regression coefficients,  $B_{0i}$  and  $B_{1i}$  are the treatment specific coefficients,  $X_{ij}$  is the independent variable, and  $e_{ij}$  is the error term. Results were considered statistically

significant at p = 0.10 due to fewer data points, although individual p values are provided for all traits so that readers can make their own interpretations of significance.

#### 3. Results

## 3.1. 2017. $CO_2 \times Water Experiment$

For  $V_{cmax}$ , species, CO<sub>2</sub>, water effects, and species × water interaction were significant accounting for 33.3, 5.8, 4.3, and 7.9% of the total variation (Table 2). INT had the greatest  $V_{cmax}$  followed by COR and ERI, with DIS having the lowest values at 44.9, 35.0, 31.4, and 24.9 µmol m<sup>-2</sup> s<sup>-1</sup>, respectively. All four willow species decreased  $V_{cmax}$  in response to eCO<sub>2</sub> (Figure 1A). Three species had lower  $V_{cmax}$  in response to DRT: COR, DIS, and ERI (Figure 1B). The significant species × water interaction resulted from INT having greater  $V_{cmax}$  in response to drought. For  $J_{max}$ , the species effect and species × water interaction were significant, accounting for 42.2 and 8.8% of the total variation (Table 2). INT had  $J_{max}$ of 133 µmol m<sup>-2</sup> s<sup>-1</sup>, which was significantly greater than the other three species that were not significantly different and averaged 82 µmol m<sup>-2</sup> s<sup>-1</sup> (not shown). Similar to  $V_{cmax}$ , the species × water interaction was due to INT, which had greater  $J_{max}$  in response to DRT, whereas the other three species had lower  $J_{max}$  in response to DRT. For *TPU*, species and water effects were significant, accounting for 33.4 and 10.6% of the total variation (Table 2). INT had the greatest *TPU* with 8.3 µmol m<sup>-2</sup> s<sup>-1</sup> and the other three were not significantly different and averaged 5.3 µmol m<sup>-2</sup> s<sup>-1</sup> (not shown).

**Table 2.** Assimilation to internal CO<sub>2</sub> traits and ANOVAs from the CO<sub>2</sub> × moisture stress treatment experiment, 2017, including source of variation, degrees of freedom (df), mean square values (MS), variance components (VC), *p*-values, and coefficient of determination ( $\mathbb{R}^2$ ). *p*-values < 0.05 are in bold print.

Source of Variation	df	$V_{cmax}$ (µmol·m <sup>-2</sup> ·s <sup>-1</sup> )			$J_{max}$ (µmol·m <sup>-2</sup> ·s <sup>-1</sup> )			<i>TPU</i> (μmol⋅m <sup>-2</sup> ⋅s <sup>-1</sup> )		
		MS	VC (%)	<i>p</i> -Value	MS	VC (%)	<i>p</i> -Value	MS	VC (%)	<i>p</i> -Value
Block	1	97.1	1.0	0.310	483.0	0.6	0.427	6.80	2.5	0.094
Species	3	1119.7	33.3	< 0.001	12059.5	42.2	< 0.001	30.54	33.4	< 0.001
CO <sub>2</sub>	1	580.6	5.8	0.016	1397.9	1.6	0.179	5.62	2.0	0.127
Water	1	431.2	4.3	0.036	1461.2	1.7	0.170	29.22	10.6	0.001
$CO_2 \times species$	3	88.0	2.6	0.422	729.1	2.6	0.414	4.26	4.6	0.155
Water $\times$ species	3	265.3	7.9	0.046	2519.2	8.8	0.027	4.43	4.8	0.142
Water $\times CO_2$	1	< 0.1	< 0.1	0.982	159.3	0.2	0.647	0.03	< 0.1	0.909
Water $\times$ CO <sub>2</sub> $\times$ species	3	79.4	2.4	0.468	361.0	1.3	0.697	1.90	2.1	0.491
Error	47	92.2	42.9		750.9	41.1		2.33	39.9	
R <sup>2</sup>				0.571			0.589			0.601

For  $A_{600}$ , the three main effects, species, water, and CO<sub>2</sub>, were significant accounting for 23.3, 20.6, and 4.9% of the total variation (Table 3). All species of  $A_{600}$  were lower in eCO<sub>2</sub> and declined on average from 14.8 to 12.1  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> or 18% (Figure 2A). All species of  $A_{600}$  were lower in DRT than WW treatments with 10.7 and 16.2,  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, respectively, a decline of 33% (Figure 2B). INT had the greatest  $A_{600}$  with 18.1 µmol m<sup>-2</sup> s<sup>-1</sup> and the other three species were not significantly different and averaged 11.9  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>, a 34% difference. For  $G_{wv600}$ , species and water effects were significant, accounting for 9.8 and 53.5 of the total variation (Table 3). There were no consistent  $eCO_2$  effects on  $G_{wv600}$  (Figure 3A); whereas drought had a strong effect in lowering the  $G_{wv600}$  on average from 0.40 to 0.10 mmol m<sup>-2</sup>s<sup>-1</sup>, a 75% decline (Figure 3B). INT had the greatest  $G_{wv600}$ with 0.36 mmol  $m^{-2 s-1}$  and the other three were not significantly different and averaged 0.21 mmol m<sup>-2</sup> s<sup>-1</sup>. For WUE<sub>600</sub>, species and water effects were significant, accounting for 7.1 and 62.3% of the total variation (Table 3). There were no consistent  $eCO_2$  effects on  $WUE_{600}$  (not shown), whereas drought had a strong effect in increasing the  $WUE_{600}$  from on average 49.7 to 143.4 or a 2.9 fold increase (not shown). COR had the greatest  $WUE_{600}$ followed by ERI and DIS, while INT had the lowest  $WUE_{600}$ .



**Figure 1.** (**A**) Maximum rate of carboxylation ( $V_{cmax}$ ) (mean  $\pm$  SE) by willow species and CO<sub>2</sub> treatments in 2017, (**B**) by willow species and moisture stress treatments in 2017, (**C**) by willow species and CO<sub>2</sub> treatments in 2018, and (**D**) by willow species and fertilization treatments in 2018. If lower case letters differ among willow species than significantly different using Tukey's mean separation test (p = 0.05).

**Table 3.** Gas exchange traits and ANOVAs from the  $CO_2 \times$  moisture stress treatment experiment, 2017, including source of variation, degrees of freedom (df), mean square values (MS), variance components (VC), *p*-values, and coefficient of determination (R<sup>2</sup>). *p*-values <0.05 are in bold print.

Source of Variation	df	$A_{600}$ (µmol·m <sup>-2</sup> ·s <sup>-1</sup> )			$G_{wv600}$ (mmol·m <sup>-2</sup> ·s <sup>-1</sup> )			WUE <sub>600</sub>		
		MS	VC (%)	<i>p</i> -Value	MS	VC (%)	<i>p</i> -Value	MS	VC (%)	<i>p</i> -Value
Block	1	46.0	2.0	0.148	0.10	6.8	0.001	2570.9	1.1	0.126
Species	3	179.1	23.3	< 0.001	0.087	9.8	0.001	5307.6	7.1	0.004
CO <sub>2</sub>	1	113.0	4.9	0.026	0.005	0.2	0.560	126.5	0.1	0.731
Water	1	474.8	20.6	< 0.001	1.422	53.5	< 0.001	140223.2	62.3	< 0.001
$CO_2 \times species$	3	18.6	2.4	0.461	0.012	1.4	0.452	1148.3	1.5	0.365
Water $\times$ species	3	15.7	2.0	0.536	0.010	1.1	0.555	1964.8	2.6	0.151
Water $\times CO_2$	1	4.6	0.2	0.644	0.041	1.5	0.094	3310.5	1.5	0.084
Water $\times$ CO <sub>2</sub> $\times$ species	3	8.6	1.1	0.751	0.009	1.0	0.572	1209.5	1.6	0.342
Error	47	21.3	43.4		0.014	24.7		1060.3	22.2	
R <sup>2</sup>				0.566			0.753			0.778



**Figure 2.** (**A**) Assimilation at 600 ppm (A<sub>600</sub>) (mean  $\pm$  SE) by willow species and CO<sub>2</sub> treatments, 2017, (**B**) by willow species and moisture stress treatments, 2017, (**C**) by willow species and CO<sub>2</sub> treatments, 2018, and (**D**) by willow species and fertilization treatments, 2018. If lower case letters differ among willow species than significantly different using Tukey's mean separation test (*p* = 0.05).





**Figure 3.** (A) Stomatal conductance at 600 ppm ( $G_{wv600}$ ) (mean  $\pm$  SE) by willow species and CO<sub>2</sub> treatments, 2017, (B) by willow species and moisture stress treatments, 2017, (C) by willow species and CO<sub>2</sub> treatments, 2018, and (D) by willow species and fertilization treatments, 2018. If lower case letters differ among willow species than significantly different using Tukey's mean separation test (p = 0.05).

# 3.2. 2018. $CO_2 \times Fertilizer Experiment$

There were no soil nutrient differences between FERT and NOFERT for organic matter, C, N, CN ratio, Ca, Na, and S (Tables 4 and 5). FERT soil nutrient traits were greater than NOFERT for K and Mg, and P. NOFERT had higher pH than FERT with 6.8 and 6.2, respectively. For  $V_{cmax}$ , species, CO<sub>2</sub>, FERT, and CO<sub>2</sub> × FERT interaction were significant, accounting for 24.1, 1.5, 61.5, and 1.6% of the total variation (Table 6). INT had the greatest  $V_{cmax}$ , followed by COR, ERI, and DIS (Figure 1C).  $V_{cmax}$  was consistently lower for all four species in eCO<sub>2</sub>. FERT increased  $V_{cmax}$  from 29.6 to 55.2 umol m<sup>-2</sup>s<sup>-1</sup> for an increase of 86% (Figure 1D). The CO<sub>2</sub> × FERT interaction was a result of a  $V_{cmax}$  downregulation under NOFERT, but upregulation under FERT treatment. For J<sub>max</sub>, species and FERT were significant, accounting for 28.1 and 54.5% of the total variation (Table 6). INT had the greatest  $J_{max}$  followed by COR, ERI, and DIS. There were no consistent CO<sub>2</sub> effects on  $J_{max}$ , but FERT showed a strongly increased average  $J_{max}$  effect of 66 and 118 umol m<sup>-2</sup>s<sup>-1</sup> for NOFERT and FERT treatments, respectively, or a 79% increase (not shown). For TPU, the effects of FERT, species  $\times$  CO<sub>2</sub>, and species  $\times$  FERT interactions were significant, accounting for 15.2, 9.4, and 9.3% of the total variation (Table 6). The significant species  $\times$  CO<sub>2</sub> interaction was a result of no consistent  $CO_2$  effect on willow species (not shown). The FERT and species  $\times$  FERT TPU interaction was the result of a FERT TPU greater than NOFERT for COR, ERI, and INT; however, DIS showed no TPU difference between FERT and NOFERT.

**Table 4.** Soil properties (mean  $\pm$  SE) during the 2018 NOFERT and FERT treatments. Treatments with different letters are significantly different using an ANOVA test,  $\alpha = 0.05$ .

Treatment	Organic	Carbon	Nitrogen	Potassium	Calcium	Magnesium	Phosphorus
	Matter (%)	(%)	(%)	(meq/100 g)	(meq/100 g)	(meq/100 g)	(ppm)
NOFERT FERT	$0.67 \pm 0.40 \text{ a} \\ 0.63 \pm 0.40 \text{ a}$	$0.39 \pm 0.02$ a $0.37 \pm 0.02$ a	$\begin{array}{c} 0.085 \pm 0.009 \text{ a} \\ 0.090 \pm 0.009 \text{ a} \end{array}$	$\begin{array}{c} 0.04 \pm 0.01 \text{ b} \\ 0.16 \pm 0.01 \text{ a} \end{array}$	$\begin{array}{c} 1.21 \pm 0.08 \text{ a} \\ 1.08 \pm 0.08 \text{ a} \end{array}$	$\begin{array}{c} 0.050 \pm 0.004 \ \text{b} \\ 0.068 \pm 0.004 \ \text{a} \end{array}$	$6.50 \pm 0.50 \text{ b}$ $9.50 \pm 0.50 \text{ a}$

Site	Sodium (%)	Sulfur (%)	pН	C:N Ratio
NOFERT FERT	$0.11 \pm 0.01$ a $0.11 \pm 0.01$ a	$0.020 \pm 0.007$ a $0.013 \pm 0.007$ a	$6.8 \pm 0.1 \text{ a} \\ 6.2 \pm 0.1 \text{ b}$	$4.7\pm0.2$ a $3.9\pm0.2$ a

**Table 5.** More soil properties (mean  $\pm$  SE) during the 2018 NOFERT and FERT treatments. Treatments with different letters are significantly different using an ANOVA test,  $\alpha = 0.05$ .

**Table 6.** Assimilation to internal CO<sub>2</sub> traits and ANOVAs from the CO<sub>2</sub> × fertilizer treatment experiment, 2018, including source of variation, degrees of freedom (df), mean square values (MS), variance components (VC), *p*-values, and coefficient of determination ( $R^2$ ). *p*-values < 0.05 are in bold print.

Source of Variation	df	$V_{cmax}$ (µmol·m <sup>-2</sup> ·s <sup>-1</sup> )		$J_{max}$ (µmol·m <sup>-2</sup> ·s <sup>-1</sup> )			TPU (µmol∙m <sup>−2</sup> ·s <sup>−1</sup> )			
		MS	VC (%)	<i>p</i> -Value	MS	VC (%)	<i>p</i> -Value	MS	VC (%)	<i>p-</i> Value
Block	1	70.5	0.4	0.160	156.0	0.2	0.424	0.80	0.1	0.746
Species	3	1366.7	24.1	< 0.001	7415.7	28.1	< 0.001	19.58	8.7	0.063
CO <sub>2</sub>	1	255.5	1.5	0.009	1.0	< 0.1	0.948	2.04	0.3	0.605
FERT	1	10471.1	61.5	< 0.001	43202.1	54.5	< 0.001	102.87	15.2	0.001
$CO_2 \times species$	3	6.5	0.1	0.904	377.1	1.4	0.208	21.36	9.4	0.048
$FERT \times species$	3	26.9	0.5	0.514	150.8	0.6	0.599	21.10	9.3	0.050
$CO_2 \times \tilde{FERT}$	1	274.0	1.6	0.007	202.6	0.3	0.362	0.45	0.1	0.809
$CO_2 \times FERT \times species$	3	41.5	0.7	0.321	195.1	0.7	0.492	10.71	4.7	0.248
Error	47	34.7	9.6		239.5	14.2		7.52	52.2	
R <sup>2</sup>				0.904			0.858			0.478

For  $A_{600}$ , species, CO<sub>2</sub>, FERT, and the CO<sub>2</sub> × FERT interaction were significant, accounting for 20.8, 1.0, 65.0, and 1.1% of the total variation (Table 7). INT had the greatest  $A_{600}$ , followed by COR, ERI, and DIS (Figure 2C). In response to eCO<sub>2</sub>,  $A_{600}$  was consistently lower under aCO<sub>2</sub> for all four species. FERT increased  $A_{600}$  from 10.4 to 19.9 µmol m<sup>-2</sup> s<sup>-1</sup> or 91% (Figure 2D). The CO<sub>2</sub>  $\times$  FERT interaction was a result of an  $A_{600}$  downregulation under NOFERT, but an upregulation under the FERT treatment. For  $G_{wv600}$ , the species, FERT, species  $\times$  CO<sub>2</sub>, and species  $\times$  FERT  $\times$  CO<sub>2</sub> interactions were significant accounting for 14.3, 43.5, 5.0, and 5.9% of total variation, respectively (Table 7). The  $G_{wv600}$ species  $\times$  FERT  $\times$  CO<sub>2</sub> interaction resulted from a varied species response to FERT and eCO<sub>2</sub> (Figure 3C,D). The species  $\times$  CO<sub>2</sub> interaction is due to rank changes, with COR and ERI decreasing in  $G_{wv600}$  response to eCO<sub>2</sub>, while DIS remained the same and INT increased  $G_{wv600}$  in response to eCO<sub>2</sub>. INT and DIS seem to respond more strongly than COR and ERI to FERT (Figure 3D). Overall, this is seen in the species response: INT and DIS having the greatest  $G_{wv600}$  response at 0.34 and 0.27 mmol m<sup>-2</sup>s<sup>-1</sup> and ERI and COR at 0.22 and 0.20 mmol  $m^{-2}s^{-1}$ , respectively (Figure 3C). The overall FERT effect was 0.161 and 0.352, for NOFERT and FERT, respectively, a 2.2 fold increase.

For 2018  $WUE_{600}$ , species and FERT effects and species × CO<sub>2</sub> interaction were significant, accounting for 19.3, 6.6, and 11.0% of the total variation (Table 7). The species × CO<sub>2</sub> interaction was a result of COR, DIS, and ERI increasing  $WUE_{600}$  in response to eCO<sub>2</sub>, whereas, INT decreased its WUE<sub>600</sub> (Figure 4A). The FERT effect was an overall decrease in  $WUE_{600}$  in response to FERT from 77.3 to 62.8 or an 18.8% decrease (Figure 4B). COR had the greatest  $WUE_{600}$  followed by ERI and INT, with DIS having the lowest  $WUE_{600}$ .

Source of Variation	df	$A_{600} \ (\mu mol \cdot m^{-2} \cdot s^{-1})$		$\frac{G_{wv600}}{(mmol\cdotm^{-2}\cdots^{-1})}$			WUE <sub>600</sub>			
		MS	VC (%)	<i>p</i> -Value	MS	VC (%)	<i>p</i> -Value	MS	VC (%)	<i>p</i> -Value
Block	1	3.39	0.2	0.411	0.001	0.1	0.743	321.3	0.6	0.448
Species	3	155.35	20.8	< 0.001	0.064	14.3	<0.001	3292.5	19.3	0.002
CO <sub>2</sub>	1	22.81	1.0	0.036	0.004	0.3	0.452	442.2	0.9	0.374
FERT	1	1454.81	65.0	< 0.001	0.588	43.5	< 0.001	3373.2	6.6	0.017
$CO_2 \times species$	3	0.84	0.1	0.916	0.023	5.0	0.042	1871.2	11.0	0.025
$FERT \times species$	3	0.91	0.1	0.906	0.015	3.4	0.125	937.4	5.5	0.178
$CO_2 \times FERT$	1	24.90	1.1	0.029	0.013	1.0	0.201	0.3	< 0.1	0.983
$CO_2 \times FERT \times species$	3	9.71	1.3	0.131	0.027	5.9	0.022	998.7	5.8	0.156
Error	47	4.92	10.3		0.008	26.5		548.5	50.3	
				0.897			0.735			0.497

**Table 7.** Gas exchange traits and ANOVAs from the  $CO_2$  x fertilizer treatment experiment, 2018, including source of variation, degrees of freedom (df), mean square values (MS), variance components (VC), *p*-values, and coefficient of determination ( $R^2$ ). *p*-values <0.05 are in bold print.





# 3.3. Covariate Relationships among Willow Species and Environmental Change

Covariate analysis of  $J_{max}$  in relation to  $V_{cmax}$ , testing CO<sub>2</sub> effect, had no significant CO<sub>2</sub> ×  $V_{cmax}$  interaction (p = 0.752). There was a significant  $J_{max}$  to  $V_{cmax}$  relationship (p < 0.001) and CO<sub>2</sub> effect (p = 0.097) (Figure 5A). Covariate analysis of  $J_{max}$  in relation to  $V_{cmax}$  testing water effect, had no significant water ×  $V_{cmax}$  interaction (p = 0.927) nor a water effect (p = 0.225). There was a significant  $J_{max}$  to  $V_{cmax}$  relationship (p < 0.001) (Figure 5 B). Covariate analysis of  $J_{max}$  in relation to  $V_{cmax}$  testing the FERT effect, had no significant FERT ×  $V_{cmax}$  interaction (p = 0.675) nor a FERT effect (p = 0.114). There was a significant  $J_{max}$  to  $V_{cmax}$  relationship (p < 0.001) (Figure 5C).

Covariate analysis of  $A_{600}$  in relation to  $G_{wv600}$  testing CO<sub>2</sub> effect, had no significant CO<sub>2</sub> ×  $G_{wv600}$  interaction (p = 0.519) nor a CO<sub>2</sub> effect (p = 0.171). There was a significant A<sub>600</sub> to G<sub>wv600</sub> relationship (p = 0.006) (Figure 6A). Covariate analysis of A<sub>600</sub> in relation to G<sub>wv600</sub> testing water effect had a significant water × G<sub>wv600</sub> interaction (p = 0.057). Thus, there were two separate slopes for WW and DRT treatments (Figure 6B). Covariate analysis of A<sub>600</sub> in relation to G<sub>wv600</sub> testing FERT effect, had no significant FERT × G<sub>wv600</sub> interaction (p = 0.509) nor a FERT effect (p = 0.249). There was a significant A<sub>600</sub> to G<sub>wv600</sub> relationship (p = 0.004) (Figure 6C).



**Figure 5.** Relationship between maximum rate of electron transport ( $J_{max}$ ) and maximum rate of carboxylation ( $V_{cmax}$ ) across four willow species under (**A**) ambient and elevated CO<sub>2</sub> treatments, 2017, (**B**) well-watered and drought treatments, 2017, and (**C**) non fertilized and fertilized treatments, 2018.



**Figure 6.** Relationship between assimilation at 600 ppm ( $A_{600}$ ) and stomatal conductance at 600 ppm ( $G_{wv600}$ ) across four willow species under (**A**) ambient and elevated CO<sub>2</sub> treatments, 2017, (**B**) well-watered and drought treatments, 2017, and (**C**) non fertilized and fertilized treatments, 2018.

Covariate analysis of  $WUE_{600}$  in relation to  $A_{600}$  testing CO<sub>2</sub> effect showed no significant CO<sub>2</sub> ×  $A_{600}$  interaction (p = 0.566) nor a CO<sub>2</sub> effect (p = 0.492). There was a significant  $WUE_{600}$  to  $A_{600}$  relationship (p = 0.049, not shown). Covariate analysis of  $WUE_{600}$  in relation to  $A_{600}$  testing water effect, had no significant water ×  $A_{600}$  interaction (p = 0.705), but a significant water effect (p = 0.007). This resulted in two separate lines of the same slope (Figure 7A). Covariate analysis of  $WUE_{600}$  in relation to  $A_{600}$  testing FERT effect, had no significant FERT ×  $A_{600}$  interaction (p = 0.370) nor a FERT effect (p = 0.183). There was no significant  $WUE_{600}$  to  $G_{wv600}$  relationship (p = 0.615, not shown).



**Figure 7.** Relationship between water use efficiency at 600 ppm and ( $WUE_{600}$ ) and (**A**) assimilation at 600 ppm ( $A_{600}$ ), 2017, and (**B**) stomatal conductance at 600 ppm ( $G_{wv600}$ ) across four willow species under well-watered and drought treatments, 2017.

Covariate analysis of  $WUE_{600}$  in relation to  $G_{wv600}$  testing CO<sub>2</sub> effect, had no significant CO<sub>2</sub> ×  $G_{wv600}$  interaction (p = 0.854) nor a CO<sub>2</sub> effect (p = 0.903). There was a significant  $WUE_{600}$  to  $G_{wv600}$  relationship (p = 0.005, not shown). Covariate analy-

sis of  $WUE_{600}$  in relation to  $G_{wv600}$  testing water effect, showed neither a significant water  $\times G_{wv600}$  interaction (p = 0.120) nor a water effect (p = 0.273). There was a significant  $WUE_{600}$  to  $G_{wv600}$  relationship in response to DRT (p < 0.001) (Figure 7B). Covariate analysis of  $WUE_{600}$  in relation to  $G_{wv600}$  testing FERT effect, showed neither a significant FERT  $\times G_{wv600}$  interaction (p = 0.448) nor a FERT effect (p = 0.752). There was no significant  $WUE_{600}$  to  $G_{wv600}$  relationship (p = 0.103, not shown).

### 4. Discussion

There were a number of consistent findings for biochemical efficiency and GE traits of these willow species between the two experiments (years). First, there was  $A_{dr}$  for all four willow species in both experiments in response to  $eCO_2$ . Second, biochemical efficiency parameters decreased for all willow species in the 2017 experiment, with reductions in  $V_{cmax}$ ,  $J_{max}$ , and TPU in response to CO<sub>2</sub>. In the second (2018) experiment, there was a reduction in  $V_{cmax}$  for all four willow species in response to eCO<sub>2</sub>. However, for  $J_{max}$ and TPU, there was no decrease, but this was probably due to the mitigation effect from fertilization, due to an increase in sink (growth) activity. Third, the willow species effect was often the most important factor accounting for between 33 and 42% of the total variation among the three traits ( $V_{cmax}$ ,  $J_{max}$ , and TPU) in the eCO<sub>2</sub> × DRT experiment and between 20 and 28% for the same three traits in the  $CO_2 \times FERT$  experiment. Fourth, in both experiments, INT had significantly greater biochemical efficiency and GE values followed by COR and ERI, with DIS having the lowest values. DIS had the lowest biomass [26] among the four species, consistent with its low biochemical efficiency values. COR had the greatest biomass, consistent with its high biochemical efficiency values. INT had intermediate biomass results, but had among the lowest leaf dry mass. In an earlier study examining three salinity treatments including control, INT had significantly greater  $V_{cmax}$ and  $J_{max}$  and GE than DIS and ERI, which were similar [15,16].

The significant species × water interaction was due to rank changes, for example,  $V_{cmax}$  was lower under DRT for COR, DIS, and ERI, but 16.5% greater for INT under DRT compared to WW. Drought response has a similar physiological effect to a saline response in that they both induce moisture stress in plants and both require cellular osmotic adjustment to withstand the stress [27]. Both stress responses require additional energy from the plant, and interestingly, INT increased its biochemical efficiency by 30% in medium salinity and high salinity, and it was the only willow species of the three tested to survive high salinity [15,16]. The greater drought and saline tolerance of INT may reflect its evolutionary origins in the arid SW USA and Mexico [28–30], where high evapotranspiration may increase drought and saline tolerance. The exact cause of the greater INT drought tolerance could be due to changes to the hydraulic architecture of the xylem and thus greater hydraulic conductivity [32] or as a result of greater osmotic adjustment [33,34].

Assimilation downregulation is the decrease in assimilation with time in response to eCO<sub>2</sub> quantified at the same measurement of CO<sub>2</sub> concentration [35,36]. There are generally at least three plausible causes recognized for  $A_{dr}$ . First, significant increases in CO<sub>2</sub> concentration often results in a decline in underlying photosynthetic capacities, carboxylation efficiencies, electron transport, and chlorophyll content [4,37,38], which is what we report on here. Second, the accumulation of nonstructural carbohydrates is usually associated with  $A_{dr}$  and is commonly interpreted as evidence of a lack of sink activity [36,39–41]. We observed a lack of sink activity in the present study, as there was no increase in stem dry mass in eCO<sub>2</sub> [26]. An increase in nonstructural carbohydrates is most likely the driver of the decline in underlying biochemical capacity. Third, it has been observed in eCO<sub>2</sub> conditions and on relatively infertile sites that limiting essential nutrients can cause  $A_{dr}$  by reducing growth [5,42].

Our preliminary data showed no  $CO_2$  effect on willow stem dry mass after the first year of growth in 2017 (p = 0.214) [26,42] found that fertilization of the eCO<sub>2</sub> plots restored eCO<sub>2</sub> growth in forest species, and [5] found fertilization reversed  $A_{dr}$ . Thus, we decided

to do a fertilizer  $\times$  CO<sub>2</sub> experiment in 2018 on a subset of fully irrigated treatments to see if we could observe a CO<sub>2</sub> effect under FERT. First, we did see a sizable eCO<sub>2</sub> effect, resulting in significant increases in biochemical efficiencies and *GE* (*A*<sub>600</sub> and *G*<sub>wv600</sub>). This change resulted in a mitigation of the biochemical downregulation found in 2017 in response to eCO<sub>2</sub>. This was doubtless driven by the FERT effect on biomass accumulation, which resulted in a significant (*p* = 0.049) increase in stem dry mass and CO<sub>2</sub>  $\times$  fertilizer interaction [26]. As in 2017, there was no stem dry mass difference between aCO<sub>2</sub> and eCO<sub>2</sub> under NOFERT in 2018, with 11.8 and 10.7 g, respectively. However, under the FERT treatment, stem dry mass was 20.0 and 25.3 g under aCO<sub>2</sub> and eCO<sub>2</sub>, respectively. Thus, nutrient limitation probably caused the lack of eCO<sub>2</sub> effect on willow growth. Subsequent soil analysis showed very low levels of nutrients under NOFERT. Also interesting was that a soil nutrient result after the FERT treatment in 2018 showed no difference between FERT and NOFERT, particularly for soil N. Thus, everything supplied by the FERT treatment was taken up by the FERT grown willows.

As mentioned, fertilization may have mitigated the biochemical downregulation in response to  $eCO_2$ , but  $eCO_2$  itself did not mitigate the DRT downregulation. Using the same willow species on a nutrient-poor former coal mine site reclamation study, we observed how green the willow foliage was. Willows apparently have a symbiotic relationship with diazotrophic endophytes in the stem that provide N to the plant in exchange for carbohydrates [43,44]. In the present study, we provided only two pulses of fertilizer early in the first year, assuming that endophytic bacteria would be present under both  $aCO_2$  and  $eCO_2$  treatments. However, it is now apparent that N fixation was either too low or that not enough other essential soil nutrients were present to allow the plants to take advantage of the  $eCO_2$  treatment.

The  $J_{max}$  to  $V_{cmax}$  species relationship under aCO<sub>2</sub> and eCO<sub>2</sub> showed the downregulation of both traits, particularly  $V_{cmax}$ , resulting in two separate lines. The INT species appear at the top of the two linear relationships and at a significant separation from the other species. The J<sub>max</sub> to V<sub>cmax</sub> species relationship under WW and DRT showed a single positive line, similar in slope to Figure 5A, but with a treatment segregation, the DRT treatments on the lower half, except for INT-D, which is at the very top of the relationship. Again, this is counter to what the other three willow species showed, but similar to the increased INT biochemical efficiency that was found in an earlier salinity study [15]. The  $J_{max}$  versus  $V_{cmax}$  relationship by species and FERT segregated primarily by treatment, though INT still showed the greatest biochemical efficiencies among the four willows under each FERT treatment. We also saw a flattening of the  $J_{max}$  to  $V_{cmax}$  relationship by one third. This may indicate a differential N allocation between the thylakoid and soluble proteins [40] or an N reallocation between photosynthetic components [5,40] examining *Picea sitchensis* and *Pinus taeda*, respectively, under differing levels of N  $\times$  CO<sub>2</sub>, did not observe a change in the *J<sub>max</sub>:V<sub>cmax</sub>* ratio, but our soils were clearly impoverished. A review by [45] showed that these two parameters are largely species-specific, which is what we observed among the four willow species under three different treatment scenarios.

Is WUE driven by changes in *A* or  $G_{wv}$  or both? It depends on the experiment. First, the  $A_{600}$  to  $G_{wv600}$  species relationship under different CO<sub>2</sub> treatments showed a single positive line with a fair amount of variation (Figure 6A). All the eCO<sub>2</sub> willows were below the single diagonal line, indicating that  $A_{600}$  decreased more in response to eCO<sub>2</sub>, and  $G_{wv600}$  remained largely unchanged in both experiments. However, WUE in the willows did not change in response to eCO<sub>2</sub>. The literature is mixed on the strength of the effect of eCO<sub>2</sub> on WUE: *Pinus radiata* had lower WUE in eCO<sub>2</sub> [3] and both pines and spruces had lower WUE in eCO<sub>2</sub> [6]. In contrast, [46] found *WUE* was greater for *Picea abies* and *Quercus rubra* grown at eCO<sub>2</sub>, and this was also the case for loblolly pine (*Pinus taeda* L.) [47]. In a study of three conifer species, *Picea mariana* and *Larix laricina* displayed a modest reduction in  $G_{wv600}$  in response to eCO<sub>2</sub>, whereas there was no response seen in *Pinus banksiana* [39] or *P. radiata* [3]. In one of the only publications on willow (*S. sagitta*) [17] response to CO<sub>2</sub> and

moisture stress is suggested that  $eCO_2$  mitigated the drought stress response in willows but not poplars. We did not find that  $eCO_2$  mitigated the response to drought stress.

In the FERT experiment, fertilization resulted in a strong increase in A and  $G_{wv}$  of 92 and 119%, which accounted for 44 and 65% of the total variation, respectively. This would be in response to the greater sink demand (growth) stimulated by the FERT treatment and the need for more assimilates. Fertilization resulted in a small, but significant decrease in WUE, but this may have been affected by very large non-specific increases in *A* and  $G_{wv}$ . The A<sub>600</sub>:Gwv<sub>600</sub> species slope was the same as the CO<sub>2</sub> species slope, but was much further extended. Nevertheless, there were no relationships of either *A* or  $G_{wv}$  with WUE, thus neither were driving the FERT WUE results from this experiment.

DRT increased the  $A_{600}$ :Gwv<sub>600</sub> species slope by a factor of three compared to the WW species slope, due mostly to the changes in  $G_{wv}$  induced by drought. Most species show a reduction of  $G_{wv}$  to control water loss during dry periods [48]. In addition, there was a four-fold reduction in  $G_{wv600}$  and the average for the four willows and water effect accounted for 54% of the  $G_{wv}$  total variation, dwarfing all other sources of variation. In contrast,  $A_{600}$  was reduced by only 50% by DRT and the water effect only accounted for 21% of the  $A_{600}$  total variation. In addition, WUE in relation to  $G_{wv}$  by species and water treatment showed a strong negative relationship ( $R^2 = 0.917$ ). Thus,  $G_{wv}$  was the primary driver and  $A_{600}$  only had a minor effect on WUE when testing under DRT. While the  $A_{600}$  to  $WUE_{600}$  relationship within water treatments (Figure 7A) might at first glance suggest that greater  $A_{600}$  results in a lower WUE, this is all driven by one species, INT. INT had lower WUE, but had greater  $A_{600}$  and  $G_{wv600}$ , showing more evidence of a strategy of increased drought tolerance and fits with the adaptive selection pressure from its evolutionary origins in the arid SW USA and Mexico [28–30]. Even in the DRT experiment, INT had greater  $G_{wv}$ than the other three willows, pointing to it being more of a drought tolerator (keeping  $G_{wv}$ more open to continue to allow A) than a drought avoider (closing  $G_{wv}$ ).

## 5. Conclusions

Willows are used as bioenergy crops often on marginal agricultural lands and for land restoration of severely disturbed sites; both site types are often nutrient-poor. Thus, with increasing atmospheric  $CO_2$  it would appear that if a site is nutrient-poor and there are no stem endophytic bacteria, the addition of soil nutrients may be necessary to sequester more available  $CO_2$ . Based on physiological and morphological results, INT is the most drought and saline tolerant of the willows tested. INT and its closest relatives in the section *Longifoliae* are also noteworthy among willow species for their ability to easily form new stems via stem suckering from a network of shallow roots, resulting in multi-stemmed colonies [49,50]. This stem-suckering feature and the drought tolerance of INT allows more  $CO_2$  capture to help mitigate climate change impacts. The rapid growth, high biomass yields, and ease of vegetative (clonal) propagation of INT from unrooted stem sections should be of interest for establishing biomass feedstock for bioenergy, and other resources for the bioeconomy, as well as for land reclamation activities on marginal land affected by drought or salinity.

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