Willow species (genus: Salix) with contrasting habitat affinities differ in their photoprotective responses to water stress

Jessica A. Savage\textsuperscript{A,D}, Jeannine Cavender-Bares\textsuperscript{B} and Amy Verhoeven\textsuperscript{C}

\textsuperscript{A}Plant Biological Sciences Program, University of Minnesota, 250 Biological Sciences Center, 1445 Gortner Avenue, Saint Paul, MN 55108, USA.
\textsuperscript{B}Department of Ecology, Evolution and Behavior, University of Minnesota, 100 Ecology, 1987 Upper Buford Circle, Saint Paul, MN 55108, USA.
\textsuperscript{C}Department of Biology, University of St Thomas, 2115 Summit Avenue, Saint Paul, MN 55105, USA.
\textsuperscript{D}Corresponding author. Email: savag070@umn.edu

Abstract. Although many Mediterranean and xeric plant species enhance their xanthophyll-mediated thermal dissipation under drought conditions, there has been limited research on photoprotective mechanism in droughted plants from other habitats. To investigate whether wetland plants utilise this mechanism under drought conditions, and whether species differ in their responses depending on their habitat affinities, we investigated the response of six willow (Salix) species to a short-term drought. In a greenhouse, 40 individuals per species were dried down over 4 weeks. Periodically during the drought, predawn and midday chlorophyll fluorescence measurements were taken and leaf discs were collected for pigment analysis with HPLC. Predawn water potential was also monitored throughout the experiment. All six species increased xanthophyll cycle activity and their capacity to dissipate excess energy during the drought by increasing their total de-epoxidised xanthophyll concentration and the concentration of zeaxanthin in proportion to chlorophyll. In general, habitat generalists had greater photoprotective responses than wetland specialists, while the wetland specialists had higher pre-drought nonphotochemical quenching. These differences are consistent with their contrasting photosynthetic rates. The observed variation in species drought responses suggests that their photoprotective strategies vary with habitat affinity.

Additional keywords: nonphotochemical quenching, wetlands, xanthophyll cycle.

Introduction

Many plant species segregate along soil moisture and larger-scale precipitation gradients based on their drought tolerance, as a result of trade-offs in physiological and life history traits that prevent them from performing well under all environmental conditions (Whittaker 1956; Brodribb and Hill 1999; Silvertown et al. 1999; Cavender-Bares et al. 2004). Although there has been substantial research investigating the role of xylem trade-offs in determining species distributions (Zimmermann and Brown 1977; Pockman et al. 1997; Grace 1995; Biehler and Fock 1996), Mehler reactions (Osmond and Grace 1995; Biehler and Fock 1996), and cyclic electron transport (Katona et al. 1992). Of these four mechanisms, the xanthophyll cycle is responsible for dissipating the majority of excess energy under drought conditions (Flexas and Medrano 2002; Demmig-Adams and Adams 2006). It is also used by plants to dissipate energy under high light, low nutrient availability, and during exposure to freezing temperatures (Adams and Demmig-Adams 1992; Demmig-Adams et al. 1995; Lovelock et al. 1995; Verhoeven et al. 1999; Cavender-Bares et al. 2005).

There is substantial evidence that Mediterranean (e.g. García-Plazaola and Becerril 2000; Kyparissis et al. 2000; Martinez-Ferri et al. 2000; Galmès et al. 2007) and xeric plant species (e.g. Balaguer et al. 2002; Barker et al. 2002) increase xanthophyll-mediated thermal dissipation under drought conditions and that tropical species utilise these processes under high light conditions (e.g. Lovelock et al. 1994; Barker et al. 1997; Watling et al. 1997; Montgomery et al. 2008).

© CSIRO 2009 10.1071/FP08303 1445-4408/09/040300
However, there has been limited research on the photoprotective responses of mesic and hydric species to drought conditions. Since photodamage is dependent on stomatal behaviour, not necessarily the severity of a drought, these species may benefit from photoprotective mechanisms if they experience prolonged stomatal closure under high light conditions (Bota et al. 2004; Flexas et al. 2006). Furthermore, if there is a cost associated with increasing xanthophyll concentrations and maintaining xanthophyll cycle activity, species may vary in their photoprotective capacity depending on the frequency and longevity of water stress they encounter in their native habitats.

To address the importance of xanthophyll mediated thermal dissipation in plants that occur in habitats with different seasonal water availability, we examined the drought responses of six willow species (genus: Salix) to a 4 week dry-down. Since willows are highly dependent on water availability (Amlin and Rood 2002; Karrenberg et al. 2002), and exhibit significant variation in habitat affinities along a soil moisture gradient (Morley 1969; Gleason and Cronquist 1991), they are an excellent system for this study. The goal of this study was to address two key questions:

1) do drought intolerant willow species exhibit enhanced xanthophyll mediated energy dissipation under drought conditions?

2) do species photoprotective responses depend on their ecological habitat of origin?

Materials and methods

Species selection

We selected six willow species (genus: Salix) native to Minnesota for our study, including three woodland specialists and three broader habitat generalists, which we classified based on habitat descriptions from Morley (1969) and Gleason and Cronquist (1991). The three woodland species (Salix candida Fluegê ex Willd., Salix pedicellaris Pursh, and Salix pyrifolia Andersson) primarily grow in fens, bogs and wet meadows in Minnesota, with S. candida inhabiting more alkaline wetlands and S. pyrifolia inhabiting more acidic wetlands. The three broader habitat generalists (Salix bebbiana Sarg., Salix discolor Muhl., Salix petiolaris Sm.) occur in a variety of habitats including prairies, moist meadows, alluvial habitats and lakeshores. They tend to occur in habitats that have more seasonal variation in water availability than the three woodland species.

Growth and dry-down conditions

In the spring of 2004, we propagated six native willow species from seed collected in south-eastern Minnesota at the Cedar Creek Ecosystem Science Reserve. We grew the willows in a greenhouse at the University of Minnesota, which was set to be 20°C year-round and achieved temperatures of 27°C on warm summer days. The plants were kept well watered and fertilised for 2 years. By the summer of 2006, plant height and stem diameter (averaged across 10 individuals per species) were 89.3 ± 3.15 cm (one standard error) and 7.60 ± 0.27 mm (s.e.), respectively. Three weeks before the start of the experiment, we transplanted the plants into 6.25-Le treepots. At this point we also measured the total leaf area of a subset of eight plants per species.

We began the dry-down treatment in June 2006. Plants were watered to field capacity and allowed to dry out over 4 weeks. We took measurements on six individuals per species, at three points in the experiment: pre-drought (day 0), mid-drought (day 15) and late drought (day 30). Plants were at field capacity during the pre-drought measurements. Plants were illuminated for 12 h per day giving a midday light intensity of ~700–800 μmol on sunny days.

Predawn water potential measurements

We measured leaf water potential (Ψ) using a Scholander pressure chamber (Soil Moisture Equipment Corp., Santa Barbara, CA, USA) 2 h before dawn each day of our measurements. We removed the leaves with razor blades, put them in plastic bags, and immediately transferred them to the pressure chamber.

Chlorophyll fluorescence and gas exchange measurements

We measured chlorophyll fluorescence on dark- and light-acclimated leaves for six individuals per species, using a pulse amplitude modulated chlorophyll fluorescence meter (LI-COR 6400–40, Li-Cor Inc., Lincoln, NE, USA). We measured minimal dark-adapted fluorescence (F₀), and maximum dark-adapted fluorescence (Fm) (using a saturating pulse of 7000 μmol m⁻² s⁻¹ for 0.8 s), in the 2-h period before dawn on one leaf per plant. We marked the spot on the leaf where the measurement was taken and measured steady-state fluorescence (Fₛ), and maximum fluorescence (Fm') on the same spot on illuminated leaves between 1300 and 1500 hours the same day. A far-red pulse (740 nm) was then applied to excite PSI and thereby oxidised PSII reaction centers for a measurement of Fₐ'. During the afternoon measurements, we also measured CO₂ assimilation at a light intensity of 1200 μmol m⁻² s⁻¹ (A₁₂₀₀). We selected this light intensity because it was greater than ambient light levels and is known to saturate photosynthesis in willows (Robinson et al. 2004). We measured the first fully-expanded, living leaf on the main stem of each plant but were unable to follow the same leaves throughout the experiment because of leaf senescence.

For our analysis, we calculated: maximum photochemical efficiency of PSII, Fm'/Fm (where Fm' = Fm - F₀); light-acclimated photochemical efficiency, ΔF'/Fm' (where ΔF = Fm' - F₀); electron transport, ETR (assuming a leaf absorbance of 0.8 and equal photon excitation of PSII and PSI); photochemical quenching, qP [(Fm' - F₀)/(Fm' - Fₛ)]; nonphotochemical quenching, qN [(Fm' - F₀)/(Fm' - Fₛ)] (Schreiber et al. 1986); and Stern-Volmer nonphotochemical quenching, NPQ [(Fm' - Fₛ)/Fm'].

Pigment analysis

Immediately after taking each chlorophyll fluorescence measurement, we punched a leaf disc from a nearby leaf with an 8-mm diameter core borer. The discs were put in microcentrifuge tubes and dropped into liquid nitrogen. These samples were kept in a −80°C freezer until they were used for pigment analysis. Pigments were extracted according to Adams and Dennen-Adams (1992). Samples were analysed by HPLC
Using an Allsphere ODS-1 (5 μm particle size, 250 × 4.6 mm) column (Alltech Chromatography, Deerfield, IL, USA). Solvents and method used are as described by Gilmore and Yamamoto (1991), however, midway through the analysis of samples the peaks began running together, so the A solvent was adjusted. The two A solvents both consisted of acetonitrile : methanol : 0.1 M Tris, pH 8.0 with a ratio of 78 : 8 : 3 for the first A solvent and 72 : 17 : 5 for the second A solvent. Adjusting the solvent altered the retention time of both chlorophylls, which were calibrated separately for each solvent. We calculated pigment concentrations on an area basis and the de-epoxidation state of the xanthophylls (DPS) as \((Z + A)/(V + A + Z)\).

**Statistics**

We used repeated-measures ANOVA to analyse pigment, chlorophyll fluorescence and gas exchange measurements overtime across species. Since there were only two individuals of *S. discolor* remaining on the last day of measurement, we presented the ANOVA analysis excluding this species. However, when we conducted the analyses including *S. discolor*, there were no qualitative differences in the results (data not shown). We used an F-test to examine the differences between habitat generalists and wetland specialists at each time interval (days 0, 15 and 30). We also used Tukey’s multiple comparisons to check for differences between species within the same habitat classification. To investigate differences in species stomatal conductance, we conducted a multiple regression analysis on the relationship between stomatal conductance and predawn water potential. Since stomatal conductance is non-linearly related to predawn water potential, we first logged the two axes. We also completed a regression analysis on species total leaf area and their decline in predawn water potential from day 0 to day 15. *P*-values < 0.05 were considered significant and values < 0.1 were considered slightly significant. All analyses were conducted with JMP 7.0 (SAS Institute, Raleigh, NC, USA).

**Results**

**Progression of dry-down**

Predawn water potential significantly decreased over time (*F*-ratio = 56.5, d.f. = 2, 18, *P* < 0.0001, Fig. 1a) during the dry-down (Fig. 1a) and this decrease corresponded with a decrease in stomatal conductance in all the species (*F*-ratio = 118, d.f. = 97, *P* < 0.0001, Fig. 1b). Across species, there was also a correlation between the decline in water potential from day 0 to 15 and their total leaf area (Fig. 1c, *F*-ratio = 14.7, d.f. = 5, *P* = 0.019). By mid-drought, the habitat generalists had significantly lower predawn water potentials than the wetland specialists (Fig. 1a), but there were no significant differences between species within the two groups. Late in the drought, the wetland specialists had achieved predawn water potentials equivalent to the mid-drought measurements of the habitat specialists. There was no habitat effect in the log–log regression of predawn water potential on stomatal conductance.

All six willow species demonstrated drought-induced senescence in response to the dry-down. In several species, the senescence occurred rapidly and multiple plants lost all their leaves by day 30. This resulted in sample sizes of 4, 6, 2, 4, 5 and 6 in the late drought for *S. bebbiana*, *S. candida*, *S. discolor*, *S. pedicellaris*, *S. pyrifolia* and *S. petiolaris*, respectively. After the experiment, we dried-down a subset of individuals from each species until they lost their leaves and then rewatered them.
Almost 60% of the plants resprouted after rewatering, but there was no difference between the resprouting ability of the habitat generalists and the wetland specialists (data not shown).

**Carbon assimilation**

All species demonstrated a significant decrease in light saturated carbon assimilation ($A_{1200}$, $F$-ratio = 44.6, d.f. = 2,18, $P < 0.0001$) and stomatal conductance ($g$) over time ($F$-ratio = 39.3, d.f. = 2,18, $P < 0.0001$). However, the habitat specialists closed their stomata more rapidly, resulting in significantly lower $g$, than the wetland specialists by the mid-drought. This resulted in lower $A_{1200}$ in the habitat generalists despite their higher initial $A_{1200}$ (Table 1).

**Chlorophyll florescence**

After the onset of the drought, photochemical quenching (qP, $F$-ratio = 16.8, d.f. = 2,18, $P = 0.0001$), light quantum efficiency ($\Delta F/F_{m}$, $F$-ratio = 245.3, d.f. = 2,18, $P < 0.0001$) and electron transport rates (ETR, $F$-ratio = 244.2, d.f. = 2,18, $P < 0.0001$) declined in all species. Although habitat generalists had significantly higher ETR predrought, their ETR declined more rapidly than the wetland specialists (Table 1). A similar trend was observed in light quantum efficiency, as $\Delta F/F_{m}$ was higher in habitat generalists than wetland specialists on day 0 but lower on day 15 (Fig. 2c, d). Meanwhile, qP demonstrated no difference between the habitat groups during the drought (Table 1).

The dark quantum efficiency ($F_{v}/F_{m}$) of PSII also significantly decreased during the drought ($F$-ratio = 79.6, d.f. = 2,18, $P < 0.0001$, Fig. 2a, b) but the decrease was small. The predrought average across species was 0.823 ± 0.002 (± s.e., n = 36) and the late drought average was 0.754 ± 0.006 (± s.e., n = 26). Habitat generalists had slightly higher $F_{v}/F_{m}$ before the drought but there were no significant differences between the two groups after the onset of the drought (Fig. 2c, d).

Across all species, there was a significant increase in nonphotochemical quenching calculated as NPQ ($F$-ratio = 7.3, d.f. = 2,18, $P = 0.005$, Table 1) and as qN ($F$-ratio = 11.6, d.f. = 2,18, $P = 0.0006$, Fig. 2e, f). Pre-drought, the wetland specialists had significantly higher NPQ and qN than the habitat generalists and this difference remained until the mid-drought in regards to qN ($\alpha = 0.5$). There were no significant differences in qN or NPQ between the species within each group.

**Pigment analysis**

All species exhibited a decline in the measured leaf pigments ($\alpha = 0.05$) during the drought and in the ratio of chlorophyll $a/b$ (Table 2). Lutein, neoxanthin and chlorophyll were significantly different between habitat generalists and wetland specialists in the late drought. However, these differences disappeared when lutein and neoxanthin were considered in proportion to chlorophyll (µmol pigment/mol chlorophyll). The ratio of $\beta$-carotene/chlorophyll did not change over time, and the ratio of lutein/chlorophyll increased during the drought ($F$-ratio = 14.8, d.f. = 2,18, $P = 0.0002$, Table 2). There was no evidence for the presence of lutein epoxide in any of the species.

Across all species, the total xanthophyll concentration (violaxanthin, antheraxanthin and zeaxanthin) in the willow leaves significantly changed over time ($F$-ratio = 9.5, d.f. = 2,18, $P = 0.002$, Fig. 3). Additionally, both the de-epoxidation state of the xanthophylls (DPS, $F$-ratio = 15.5, d.f. = 2,18, $P = 0.0002$, Fig. 3) and the ratio of zeaxanthin/chlorophyll significantly increased ($F$-ratio = 34.2, d.f. = 2,18, $P < 0.0001$, Fig. 4). The increase in DPS correlated linearly with an increase in qN (Fig. 5a) and NPQ (Table 1), and was greater habitat generalists than wetland specialists (Fig. 5b). Habitat generalists also had significantly higher ratios of zeaxanthin/chlorophyll and violaxanthin/chlorophyll (Fig. 4) during the mid-drought. However, the ratio of antheraxanthin/chlorophyll did not change over time ($F$-ratio = 4.1, d.f. = 2,18, $P = 0.05$, Fig. 5c).

### Table 1. Average chlorophyll fluorescence and gas exchange parameters of six different willow species during the dry down

<table>
<thead>
<tr>
<th>Day</th>
<th>S. bebbiana</th>
<th>S. discolor</th>
<th>S. petiolaris</th>
<th>S. candida</th>
<th>Wetland specialists</th>
<th>S. pyrifolia</th>
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Table 2. Average leaf pigment concentrations of six different willow species during the dry down

Pigments are indicated by the following abbreviations: neoxanthin (neo), lutein (lut) and β-carotene (β-c). There were no significant differences between habitat generalists and wetland specialists in these pigment concentrations. Pigments that significantly (α = 0.01) changed during the drought based on a repeated-measures ANOVA where n = 26 are marked, *. Values are reported ± s.e.

<table>
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<tr>
<th>Day</th>
<th>S. bebbiana</th>
<th>S. discolor</th>
<th>S. petiolaris</th>
<th>S. candida</th>
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Fig. 2. (a, b) Maximum photochemical efficiency of PSII, \( F_v/F_m \), (c, d) light-acclimated photochemical efficiency, \( ΔF/F_m \), (c, f) and nonphotochemical quenching, \( qN \), differed between wetland specialists (open symbols) and habitat generalists (closed symbols) during the drought. Error bars are ± s.e.; * indicates where the habitat generalists significantly differ from the wetland specialists (α = 0.05). The reported values are the species averages are ± s.e. Species symbols are the same as Fig. 1.
chlorophyll was only significantly higher in the habitat generalists late in the drought ($P = 0.03$, Fig. 4). The greater DPS values of the habitat generalists in both the mid-($P = 0.07$) and late drought ($P = 0.08$, Fig. 4) were slightly significant, indicating that the habitat generalists may have greater xanthophyll cycle activity than wetland specialists. This relationships was stronger when species were compared at similar predawn water potentials (habitat generalists, day 15 and wetland specialists, day 30) ($P = 0.02$).

When considered individually, the six willow species demonstrated distinct responses to the imposed drought treatment. While *S. discolor* exhibited the greatest increase in zeaxanthin both in absolute concentration and concentration in proportion to total chlorophyll, *S. pyrifolia* and *S. pedicellaris* demonstrated relatively small changes in zeaxanthin concentration (Fig. 4). Three of the species (*S. bebbiana*, *S. discolor* and *S. candida*) also increased their total xanthophyll concentrations by mid-drought (Table 2).
Photoprotection and the xanthophyll cycle

This experiment demonstrated that six willow species which are relatively drought intolerant can dissipate excess energy through the xanthophyll cycle in response to drought conditions. During a 4 week dry down, these species exhibited both an increase in their de-epoxidated xanthophyll concentrations (DPS) and their nonphotochemical quenching (qN, Figs 2, 3, 5). Further, some of the species increased their capacity to dissipate excess energy by upregulating xanthophyll production. This is significant because leaf light absorption declines with chlorophyll level and an increase in the proportion of zeaxanthin to chlorophyll (Fig. 4) enhances a plant’s photoprotective capacity.

All six of the species in this study were able to minimise photodamage and maintain high maximum photosynthetic efficiency (Fv/Fm, Fig. 2a, b) during the drought in some of their leaves. However, most of the species also exhibited significant leaf loss and senescence. This is important to note because high light is known to shorten leaf life span in many species (Williams et al. 1989; Cavender-Bares et al. 2000), and there is evidence that photodamage may play a role in leaf senescence (Lovelock et al. 1994). It is, therefore, possible that the species in this study did experience photodamage during the dry down but it went undetected because we do not have measurements immediately before senescence on individual leaves.

Leaf chlorophyll loss

All six species in this study demonstrated a decline in leaf chlorophyll content during the 4 week dry-down. Chlorophyll loss is common under drought conditions in other deciduous species (Munné-Bosch et al. 2001), and also some perennial grasses (Balaguer et al. 2002) and evergreen species (Martínez-Ferri et al. 2000; Munné-Bosch and Alegre 2000). Although chlorophyll loss can result from oxidative damage, there is increasing evidence that it can also result from enzyme-mediated processes (Matile et al. 1999). These processes may be advantageous as they reduce light absorption and can limit the amount of damaging excess energy in the leaf (Adams et al. 1990; Munné-Bosch et al. 2001). We note that all six willow species exhibited a slower degradation of chlorophyll a than chlorophyll b over time. This pattern has been observed in several other senescing species (Biswal 1995; Suzuki and Shioi 2004) but is not ubiquitous (Munné-Bosch et al. 2001).

Drought deciduousness and photoprotection

Xanthophyll cycle activity and chlorophyll loss played an important role in the photoprotective responses of these six willow species during the dry-down, but these processes were only effective in preventing leaf damage under mild to moderate drought conditions. After 4 weeks of drought, many of the plants began to senescence. Although this senescence was not lethal, and many plants resprouted after rewatering, it did indicate that these species rely on other mechanisms to survive more severe droughts.

Plant drought response strategies are generally broken into two categories: drought avoidance and drought tolerance. Some plants ‘avoid’ drought conditions and high xylem tensions by rapidly closing their stomata, and other plants ‘tolerate’ drought conditions by maintaining function at low water potentials. The six willows in this experiment are drought avoiders, which is similar to other early successional species (Martínez-Ferri et al. 2000). Consistent with other drought avoiders, they minimise photodamage by reducing their leaf chlorophyll levels, and limit water loss by dropping their leaves (Martínez-Ferri et al. 2000; Munné-Bosch and Peñuelas 2003; Munné-Bosch and Alegre 2004). Willows are also effective drought avoiders because they are avid resprouters (Newsholme 1992; Karrenberg et al. 2002), and some species can refill cavitated vessels (Utsumi et al. 1998).

The ecological significance of species responses

The habitat generalists (S. bebbiana, S. discolor, and S. petiolaris) and the wetland specialists (S. candida, S. pedicellaris, and S. pyrifolia) demonstrated significant differences in their function even before the initiation of the drought treatment. The wetland specialists, on average, had lower predrought photosynthetic activity (A1200), light-acclimated photochemical efficiency (ΔF/Fm′) and overall leaf area, and higher qN and DPS than the habitat generalists (Table 1, Figs 1, 2). These differences...
may be indicative of contrasting growth strategies in the species. In general, plants from nutrient limited systems such as wetlands are known to have more conservative growth strategies than species in more productive environments (Grime and Hunt 1975; Chapin 1980; Reich 1993). If this is the case with willows, then differences in species predrought and drought physiology could be related to their distinct growth strategies. It is also possible that wetland plants specifically benefit from higher levels of qN and DPS under well watered conditions because of the greater chance of waterlogging in their native habitats. Since waterlogging causes a reduction in photosynthesis, it can also lead to photodamage in some plants (Close and Davidson 2003).

After the initiation of the drought, the habitat generalists and wetland specialists continued to diverge in physiological function. The habitat generalists greater leaf areas likely contributed to their large declines in predawn water potential by mid-drought (Fig. 1c). At that point, the habitat generalists were demonstrating more conservative water use and lower rates of stomatal conductance (g) than the wetland specialists. This resulted in lower $A_{1200}$ and electron transport (ETR) in these species (Table 1). Although the wetland specialists maintained greater photosynthetic function into the mid-drought, it is possible that under drought conditions, willows benefit from dormancy. In general, the habitat generalists senesced before the wetland specialists and by the end of the dry-down, 39% of the habitat generalists had lost all of their leaves. When the plants were rewatered, there were no significant differences in the resprouting ability of the wetland specialists and the habitat generalists, however, we only investigated resprouting immediately after senescence and it is possible that species exhibit different responses after longer periods of dormancy.

Another difference in the drought responses of the habitat generalists and the wetland specialists involved their photoprotective activity. The habitat generalists, on average, exhibited the greatest photoprotective responses to the drought, as indicated by their larger increases in both qN and DPS (Fig. 5). Although the habitat generalists’ lower predawn water potentials in the mid-drought can partially explain their higher photoprotective response, the wetland specialists still failed to increase their xanthophyll activity in the late drought when they had achieved comparable predawn water potentials. These results indicate that the wetland specialists exhibit smaller or slower photoprotective responses to drought than the habitat generalists, suggesting that there is a relationship between species photoprotective ability and their habitat affinity.

The six willow species in the study also demonstrated differences in their carotenoid concentrations during the drought, but these differences did not correspond with their habitat classifications. Since many carotenoids such as lutein and β-carotene are involved in thermal quenching and scavenging of reactive oxygen species (Dall’Osto et al. 2006), differences in their concentrations may be important to species drought tolerance. Furthermore, some plants use other antioxidants including salicylic acid, to minimise photodamage under drought conditions (Yang et al. 2004; Abreu and Munné-Bosch 2007). Therefore, it is possible other pigments and antioxidants besides those measured are important to these species drought response, but further research is needed to better understand these processes in willows.

Conclusion
Willows generally inhabit mesic and hydric habitats and are considered drought intolerant compared with many other plant species. Although willows rarely encounter severe droughts in their native habitats, they often encounter changes in water availability that can reduce their stomatal conductance (Pockman and Sperry 2000; Amlin and Rood 2002), making them susceptible to photodamage under conditions of excess light. This experiment demonstrated that willows are capable of increasing their xanthophyll mediated thermal dissipation under drought conditions, and that the rate and extent of their response appears to vary with their habitat affinity.

Acknowledgements
We thank Cassandra Olson and Ethan Warner for assisting with data collection. All the seeds for this experiment were collected at Cedar Creek Ecosystem Science Reserve and we appreciate John Haarstad’s and Barbara Delaney’s assistance in locating the species at the site. We also would like to thank Dustin Haines, Rebecca Montgomery, Peter Reich, George Weiblen, and Ruth Shaw and for their feedback on the paper and experiment. Funding was provided by a National Science Foundation LTER grant to the Cedar Creek Ecosystem Science Reserve (DEB: 0620652, JCB), Dayton Natural History Funds (JS) and the University of Minnesota, Department of Plant Biology (JS).

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Manuscript received 27 November 2008, accepted 18 February 2009