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Susceptibility of *Salix monticola* to Cytospora canker under increased temperatures and decreased water levels

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ABSTRACT

In the past two decades, a large-scale decline of tall riparian willow populations has occurred in parts of the Rocky Mountains, USA. Previous research has demonstrated that the biotic factors ungulate browsing and *Valsa sordida* (*Cytospora chrysosperma*), a fungal infection, are drivers of the decline. Increased air temperatures and decreased water levels as predicted by climate models may interact with biotic factors to produce further decline. To examine the effects of potential climate change on willows, we implemented a factorial field experiment in Rocky Mountain National Park, Colorado. We measured the effects of high and low water levels, increased air temperature, and fungal infection on above and belowground willow biomass. There was no significant difference in aboveground biomass under any treatment. Warmed treatments resulted in significantly greater belowground biomass. Inoculated stems were highly susceptible to potentially lethal fungal cankers under all treatments. This suggests that willows presently are highly sensitive to Cytospora canker and future climate changes may have little additional effect on their susceptibility to fungal infection. Cytospora canker can lead to stem death and resource managers should work to reduce mechanisms of stem wounding, such as ungulate browsing, that may allow entrance for fungal infection and lead to further riparian willow dieback.

1. Introduction

Climate-driven declines in species abundance have occurred across the world (Allen et al., 2010), altering processes such as nutrient cycling and energy and water fluxes, resulting in cascading effects through ecosystems (Anderegg et al., 2012b). Climate-related stressors may decrease a plants ability to defend itself against diseases (Ayres, 1984; Boyer, 1995). For example, drought is recognized as a potential predisposing factor for pathogen infection (Desprez-Loustau et al., 2006; Manion and Lachance, 1992) and climate change has increased the risk of pathogen outbreaks (Burdon et al., 2006; Garrett et al., 2006; Harvell et al., 2002). Widespread aspen (*Populus tremuloides*) decline in western North America has been linked to extreme drought, above average temperatures, fungal pathogens and insect attacks (Anderegg et al., 2012a; Michaelian et al., 2011; Worrall et al., 2013). Dieback of thinleaf alder (*Alnus incana* ssp. *tenuifolia*), a common riparian tree in western North America, is reported to be triggered by high maximum summer temperatures and an epidemic canker caused by *Valsa melanodiscus* (*Cytospora umbrina*) (Worrall et al., 2010).

Large scale dieback and decline of riparian Salix spp. (willow) communities has been documented in parts of the Rocky Mountains over the past two decades, including Montana, Wyoming and Colorado (Limb et al., 2003; Marshall et al., 2013; Peinetti et al., 2002). For example, in Yellowstone National Park, willow dieback has been attributed to elk browsing and an absence of beavers (Bilyeu et al., 2008; Marshall et al., 2013). In Rocky Mountain National Park, Colorado (RMNP) willows have 2–4 m tall dead stems, while most live stems are <1 m tall. The dead stems did not result from climatic events, but from a complex interaction among sapsuckers, *Valsa sordida* (*Cytospora chrysosperma*, hereafter referred to as Cytospora canker) infection, and ungulate browsing (Kaczynski, 2013).

Cytospora canker is a world-wide disease that occurs on at least 85 host woody plant species (Sinclair et al., 1987). In the Rocky Mountains, Cytospora canker affect a wide range of horticultural and native species, including apples (*Malus* spp.), plums (*Prunus* spp.), birches (*Betula* spp.), willows (*Salix* spp.), aspens and poplars (*Populus* spp.) (Jacobi, 1994). In Colorado, 97% of sampled native aspen stands had Cytospora canker present (Hinds, 1964). The prevalence of Cytospora canker in willow stands in unknown.

Riparian willows are phreatophytes that utilize shallow groundwater. Drought stress in willows can develop from two processes: (1) reduced stream flow created by low precipitation or (2) the

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absence of beavers, either of which can reduce ground water levels. In the Rocky Mountains, stream flow is dominated by snowmelt, and low flows occur following winters with a low water content snowpack (Hauer et al., 1997). Beavers utilize tall woody riparian vegetation as a food source and for dam building and they can control local water tables. Beaver dams have been shown to moderate the hydrologic effects of climate (Westbrook et al., 2006), allowing willows access to shallow late summer groundwater. However, beavers cannot occupy stream reaches dominated by short willows, as they do not provide adequate dam building material.

Increasing air temperature has created earlier peak stream flows leading to reduced late summer flows throughout the Rocky Mountains (Clow, 2010; Stewart et al., 2005; McGuire et al., 2012). Under projected climate change scenarios, mean annual temperature is projected to increase by 3 °C in middle elevation zones in the Rocky Mountains, increasing the length of summer (Bradley et al., 2004). More precipitation is falling as rain during the winter months, reducing snow accumulation and producing lower summer stream flows (Knowles et al., 2006). These changes can result in increased plant drought stress, particularly if beavers are absent. In addition, warming winter temperatures may facilitate fungal infections while plants are dormant and cannot fight the infection (Harvell et al., 2002).

Field and greenhouse studies have demonstrated that woody plants weakened by drought and then wounded (Guyon et al., 1996; Kepley and Jacobi, 2000) or exposed to warming (Bitty et al., 2004), are more susceptible to a wide range of pathogen infections. In this paper we examine the effects of experimentally warmed summer air temperatures and manipulated water table depth to simulate drought stress on the tall riparian willow species *Salix monticola*. We evaluate how above and belowground willow growth and leaf level stomatal conductance respond to these stressors and influence the initiation of Cytospora canker on stems. We use our factorial field experimental results to answer three questions: (1) Are willows with reduced water availability more susceptible to fungal infection? (2) Do higher air temperatures make willows more susceptible to fungal infection? and (3) How does Cytospora infection affect above- and belowground biomass?

2. Material and methods

The experiment was implemented in Rocky Mountain National Park (RMNP), Colorado (40.33° N, 105.60° W; elevation: 2620 m). We analyzed *S. monticola*, the most abundant tall (2.5–4.5 m in height) riparian willow in RMNP. Willow stems were collected from randomly selected plants while dormant in January 2011. Stems were cut to approximately 60 cm length and ranged from 10.4 to 20.1 mm in diameter. Stems were kept in cold storage until April, 2011, then planted in a mixture of potting soil (Premier Horticulture Pro-mix BX Mycorrhizae) and sand to create a sandy loam similar to floodplain soils in the study area (Westbrook et al., 2006). Each stem was planted 10 cm into the soil in a 2.83 L (10 cm × 36 cm) tree pot (Stuewe and Sons, Tangent, OR, USA) and eight pots were put into each of twelve 114 L rubber bins (N = 96 willow stems). Stems were grown in a greenhouse at Colorado State University in Fort Collins, CO (elevation: 1525 m), hardened through exposure outside of the greenhouse in mid-May to acclimate and transported to RMNP in early June 2011. The experiment was conducted from June through August, 2011. The willow stems were then harvested at the end of the growing season.

2.1. Experimental design

We used a split–split plot experimental design using temperature as a whole plot factor, ground water level as a subplot factor, and inoculation of Cytospora canker as a sub-subplot factor. We built three passive warming shelters using clear poly vinyl plastic. Each warming shelter was paired with an ambient temperature control and all were covered with clear plastic corrugated roofing (Suntuf™) to exclude precipitation (Fig. 1). Wooden posts were used as corner supports. A univent automatic opener was installed on each warming shelter to open or close the roof to maintain a temperature 2–3 °C above ambient. Temperatures were recorded hourly in the warming treatment and ambient control (n = 6) using Hobo H8 units (Onset) (Fig. 2). Within each temperature treatment, two water level treatments were used to simulate a shallow (23 cm depth to water) and deeper (40 cm depth to water) water table. Water levels were controlled with holes in the rubber bins that limited the highest water table depth in each bin. Plants were watered weekly until water flowed out the bin holes. Percent

Fig. 1. One replication of the experiment: ambient temperature in front, warming shelter in plastic sheeting behind.

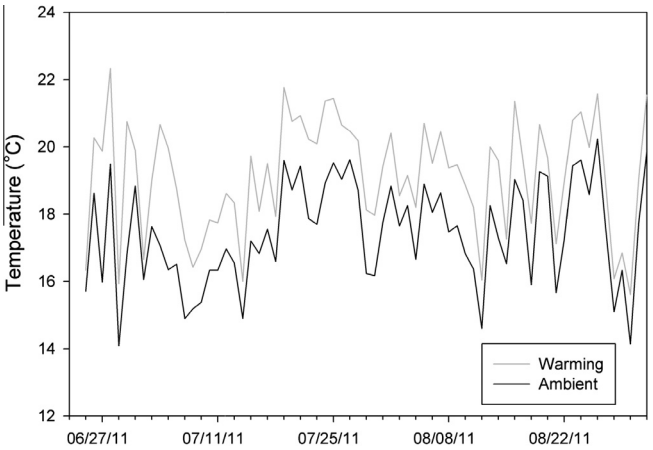

Fig. 2. Daily mean temperature differences between warming treatment and ambient control. Means are averaged for the three warming treatments and three ambient controls.

volumetric soil water content, on a scale of 0% (air dry) to 100% (saturated), was measured biweekly with a 12 cm total domain reflectivity (TDR) probe (Campbell Hydrosense®).

2.2. Fungal isolates

Two isolates of *V. sordida* were used in the experiment. Live willow stem sections with fruiting fungal pycnidia were collected from RMNP, placed in humidity chambers to force spore production, and spores were cultured on ¼ strength potato dextrose agar (PDA) ++ media (with antibiotics Streptomycin sulfate (0.1 g/5 ml of H_2O) and Chloramphenicol (0.1 g/2.5 ml ethanol) per 500 ml of liquid media). DNA of the isolates was analyzed using Easy DNA™ kit Genomic DNA isolation (Invitrogen corp) methods (protocol #3) to extract DNA from 10 day old fungal cultures. Polymerase chain reaction (PCR) amplification techniques were performed using Internal Transcribed Sequence (ITS) universal primers 1 and 4 (White et al., 1990). Sequencing was performed at the Proteomics and Metabolomics Facility at Colorado State University. Sequences were matched to known *Valsa* spp. (*Cytospora* spp.) sequences (from the laboratory of Dr. Gerald Adams, Department of Plant Pathology, Michigan State University), and were identified as *V. sordida* (*C. chrysosperma*). Isolates were then transferred to petri dishes containing full strength PDA and grown in the laboratory at 23 °C for one week prior to inoculation into the study willows.

2.3. Inoculations

Willows were grown in the field under full treatment conditions for 7 days prior to inoculation in late June 2011. Four of the eight stems in each bin were randomly selected for inoculation ($n = 48$). The bark was wiped with 95% ethanol then three circular 7 mm diameter wounds, approximately 10 cm apart, were created using a leather punch to remove the bark and cambium. The two lowest wounds were inoculated with a 7 mm PDA with *V. sordida* plug, while the top wound was a control inoculated with sterile PDA. Wounds were wrapped in parafilm for 7 days after inoculation to prevent desiccation. Vertical canker growth was measured in late August 2011. Canker length was compared between both main effects and interactions using ANOVA.

2.4. Plant responses

Current annual aboveground (AG) and belowground (BG) biomass were compared between treatments. AG biomass was calculated using new shoot length and diameter on all stems. Fifty-five shoots were collected to develop quantitative relationships between shoot length (cm), diameter (mm) and biomass (g) using regression analysis (methods from Bilyeu et al., 2007). The linear regression relating shoot length to biomass was used to determine AG biomass ($r^2 = 0.94$, biomass: diameter, $r^2 = 0.86$). Mass was transformed to linearize the data and achieve homogeneity of variance, where $\ln(\text{biomass}_i + 0.85) = 0.0188 * \text{length}_i - 0.1245$. Small shoots infrequently had negative estimates of mass and were assigned a mass of 0.01 g.

BG biomass was measured by drying and weighing roots. All plants were harvested and the stem was removed. We washed all soil from roots and dried roots at room temperature for 4 months. All BG biomass was 0 g at the start of the year because bare willow stems were planted.

Stomatal conductance was measured on a representative leaf on each plant in mid August using a Li-Cor 6400XT. Photosynthetic active radiation (PAR) was held constant at 1500.

2.5. Statistical analyses

Treatment main effects and interactions on BG biomass, AG:BG ratio (using post treatment AG production), and canker growth were analyzed using a generalized linear model with a random effect for replicate. Effect size (ES) was also computed when there was a significant treatment effect. Final AG biomass was analyzed using ANCOVA, with pre-treatment AG biomass as a covariate. All analyses were completed using Rx64 v2.15 (R project.org) using the nlme package (Pinheiro et al., 2013). Stomatal conductance measured once during the experiment was investigated only for main effects.

3. Results

3.1. Warming/ambient and shallow/deep water table treatment effects

Soil moisture was significantly lower in the deep water table treatment compared with the shallow water table ($p < 0.001$; Fig. 3). Post-treatment AG biomass was not significantly different between the shallow and deep water table treatments ($p = 0.35$), or between the warming and ambient temperature treatments ($p = 0.283$), when accounting for pre-treatment AG biomass (Table 1). There was no interaction between water or temperature treatment on AG biomass ($p = 0.878$).

BG biomass in the warming treatments was 30% greater than ambient BG biomass ($p = 0.012$; ES = 0.065) (Table 1). BG biomass was not significantly different between the shallow and deep water table treatments ($p = 0.313$), and there was no interaction between temperature and water on BG biomass ($p = 0.191$).

The ratio of AG:BG biomass was not significantly different between ambient and warming treatments ($p = 0.0525$), nor water levels ($p = 0.227$) (Table 1). The interaction of water and temperature was not significant ($p = 0.086$).

Plants in the deep water table treatment had lower stomatal conductance than shallow water table plants, 0.10 mol H_2O/m^2s compared with 0.14 mol H_2O/m^2s ($p = 0.0031$; ES = 0.0896) (Table 1). Stomatal conductance did not differ between plants in the warmed treatment and control ($p = 0.768$).

3.2. Effect of drought and warming on Cytospora canker

Wounds inoculated with Cytospora had a 95% infection rate. Cytospora cankers averaged 45 mm and 52 mm in length on the

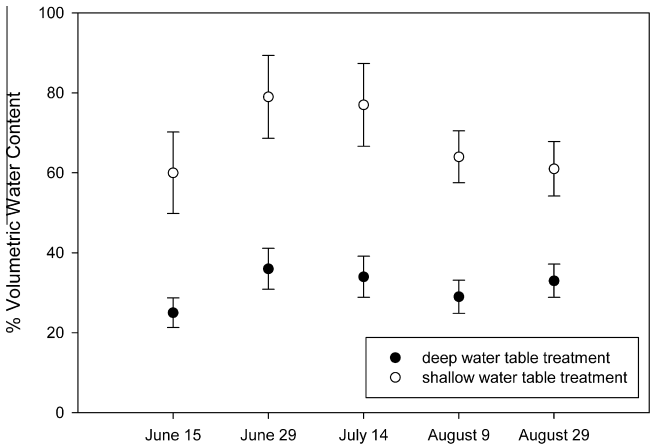

Fig. 3. Average soil moisture (volumetric water content) at 12 cm in shallow (well watered) and deep (drought) treatments with 95% confidence intervals. Shallow and deep water soil moisture is significantly different on all dates ($p < 0.001$).

Table 1
Mean biomass and stomatal conductance for main effects for treatments and controls.

	AG-post (g)	BG (g)	AG:BG	Stomatal conductance (mol H_2O/m^2s)
Inoculated	8.30 (+/−1.18)	24.38 (+/−3.44)	0.39 (+/−0.13)	0.11 (+/−0.02)
Control	6.31 (+/−0.87)	25.16 (+/−3.85)	0.39 (+/−0.07)	0.13 (+/−0.02)
Warming	7.14 (+/−1.02)	28.04 (+/−3.94)*	0.31 (+/−0.06)	0.12 (+/−0.02)
Ambient	7.47 (+/−1.11)	21.50 (+/−3.07)*	0.46 (+/−0.14)	0.12 (+/−0.02)
Drought	6.87 (+/−0.70)	23.22 (+/−3.71)	0.44 (+/−0.14)	0.10 (+/−0.02)**
Watered	7.72 (+/−1.32)	26.1 (+/−3.56)	0.34 (+/−0.06)	0.14 (+/−0.02)**

* Significantly different at 0.05 level. No interactions were significant. 95% confidence interval in parentheses.
** Significantly different at 0.01 level.

Table 2
Mean vertical canker length for each treatment: warming, ambient, reduced water and watered. All elongated cankers from inoculated wounds were significantly larger than controls. 95% confidence interval in parentheses.

	Inoculated wound (mm)	Control wound (mm)
Warming	51 (+/−11.67)	11 (+/−0.27)
Ambient	48 (+/−11.19)	10 (+/−1.85)
Reduced water	51 (+/−11.58)	10 (+/−0.30)
Watered	52 (+/−11.18)	11 (+/−1.56)

middle and bottom wound and were significantly larger than the necrosis around the control wounds that averaged 11 mm (wound 1 vs. control: $p < 0.000$; wound 2 vs. control: $p < 0.000$). There were no significant differences in canker lengths between the two isolates ($t = 0.266$, $p = 0.791$) or the wound position ($t = -1.959$, $p = 0.054$), therefore the isolate and wound number effects were combined (Table 2). There was no significant difference between canker lengths on plants in warming treatment vs. ambient ($p = 0.594$), shallow water vs. deep water table treatments ($p = 0.346$) or the interaction of temperature and water ($p = 0.73$). Stomatal conductance averaged 0.11 mol H_2O/m^2s in leaves on inoculated plants and 0.13 mol H_2O/m^2s ($p = 0.085$) in control plant leaves (Table 1).

3.3. Cytospora effects on AG and BG biomass

Inoculations had no effect on either AG ($p = 0.149$) or BG biomass ($p = 0.768$). There were no significant effects on BG biomass due to the interaction between temperature and inoculation ($p = 0.371$), water and inoculation ($p = 0.576$) and the three way interaction, between water, temperature and inoculation, ($p = 0.563$). When accounting for pre-treatment biomass, there were no significant effects on AG biomass from the interaction between water and inoculation ($p = 0.549$), temperature and inoculation ($p = 0.443$) or a three-way interaction ($p = 0.626$). Inoculations had no effect on the ratio of AG:BG biomass ($p = 0.892$). The interaction of water and inoculation ($p = 0.597$), temperature and inoculation ($p = 0.508$) and the three way interaction ($p = 0.387$) were all not significant.

4. Discussion

Riparian willow dieback in the Rocky Mountains has been linked to biotic factors including ungulate browsing, sapsucker wounding and Cytospora infection (Kaczynski, 2013; Singer et al., 1998). Drought, caused by the absence of beavers, decreased stream flow and increased air temperature are reported to increase plant susceptibility to pathogen outbreaks (Guyon et al., 1996; Kepley and Jacobi, 2000). However, we found that inoculated willows in all treatments and controls were highly susceptible to Cytospora infection that produce potentially lethal cankers.

4.1. Cytospora canker infection

Previous research has suggested that *V. sordida* can be endophytic and latent within healthy *Populus* spp. stems (Christenson, 1940). When the stem dried, fruiting bodies of the fungi became abundant, suggesting that in times of stress, the host can be susceptible as the pathogen becomes active, potentially killing the stem (Christenson, 1940). Chapela (1989) isolated *V. sordida* from 27% of moderately dried branch samples across 11 aspen trees. In contrast, McIntyre et al. (1996) studied the possibility of *V. sordida* growing inside the bark of live aspen trees and did not find the pathogen on any of the 349 samples. The issue of endophytic *V. sordida* has not been thoroughly studied in willows.

4.2. Reduced water availability and warming effects on Cytospora cankers

A number of researchers have reported that drought stressed plants are more prone to Cytospora cankers (Kepley and Jacobi, 2000; McIntyre et al., 1996). However, our research demonstrated that reduced water availability did not significantly increase the incidence or severity of cankers in inoculated willow plants. Cankers were not significantly different in length between the two water treatments, indicating the high potential susceptibility of willow Cytospora infection. Previous research in RMNP demonstrated that 57% of wounded and field inoculated willow stems with canker expansion had low predawn xylem pressure potentials in the summer, suggesting that well even watered plants were susceptible to fungal infection (Kaczynski unpublished data). Siberian alder (*Alnus viridis* ssp. *fruticosa*) are susceptible to *C. umbrina* cankers at both high and low water stress (Rohrs-Richey et al., 2011). Aspen exhibits increased incidence of fungal infection during periods of drought stress (McIntyre et al., 1996), however this response may not be generalized within the family Salicaceae.

Increased air temperatures have been implicated in the rapid decline of thinleaf alder due to infection with *C. umbrina* (Worrall et al., 2010). However, previous studies have not used an experimental approach to analyze the effects of warming and Cytospora infection. Experimental warming may produce a similar plant response as drought, but responses are species specific (Bitty et al., 2004). A 2–3 °C increase in temperature did not increase the susceptibility of *S. monticola* to Cytospora canker when compared to the controls, and inoculated wounds had significantly larger cankers than control wounds.

Our inoculations reduced willow stomatal conductance in August, a period of the summer characterized by late summer drought, by 15%, although this was not a significant difference. Rohrs-Richey et al. (2011) found that water stress and Cytospora canker reduced stomatal conductance by 40% on Siberian alders. Inoculations and subsequent canker growth had no significant negative effects on willow growth as measured by AG biomass, BG biomass, and AG:BG biomass. Once Cytospora spores enter the host the cambium and adjacent tissues are infected resulting in stem

death (Biggs and Davis, 1983). A longer duration experiment could have resulted in greater effects on AG and BG biomass, however we have observed that while the initial effects of a Cytospora canker are limited, 93% of infected stems perish prior to the next growing season (Kaczynski, 2013).

4.3. Belowground biomass and current annual growth

Willow belowground biomass was significantly greater in the warming treatment vs. the controls. Warming has been shown to increase root biomass regardless of water level in many ecosystems (Gill and Jackson, 2000; Pendall et al., 2004; Wu et al., 2011). As air temperatures rise, soil temperatures are also expected to rise (Schlesinger and Andrews, 2000), and root growth is positively correlated with soil temperature, when variables such as nutrients and water are controlled (Pregitzer et al., 2000). In Alaska, experimental warming on *Salix rotundifolia* produced a 25% increase in belowground biomass compared with plants in ambient temperatures (Hollister and Flaherty, 2010).

Warming increases initial root growth, however root turnover can also increase, leading to long term reductions in root biomass (King et al., 1999; Pregitzer et al., 2000). Under warming conditions, *P. tremuloides* clones had greater root production and mortality than controls (King et al., 1999). Our results on *S. monticola* confirm this short-term response in root growth. This increased root growth may be particularly important because it would facilitate the growth of roots into deeper soils allowing continued access to ground water, that phreatophytes like *S. monticola* require.

There was no significant difference in AG biomass or the ratio of AG:BG biomass produced by either the warming or water level treatments. Short-term experimental warming of woody plants, such as *S. rotundifolia*, have not resulted in significant changes in annual net primary production or growth (Hollister and Flaherty, 2010). A 10-year field experiment in Yellowstone NP showed that higher water tables result in greater willow net primary production, and significant effects were measured within 3 years (Bilyeu et al., 2008 and Marshall et al., 2013).

5. Conclusions and implications

V. sordida is a native fungus known for nearly 100 years to infect willows (Long, 1918). Warming and reduced water treatments, consistent with climate change scenarios, appeared to have little effect on willow fungal infection rates. Although our experiment was conducted over one growing season it demonstrated that once present on a wound, Cytospora cankers expanded under diverse environmental conditions. Because Cytospora infection typically results in stem death once infection occurs, stem death is likely.

Resource specialists should manage for both biotic and climate drivers that can influence willow dieback. Biological factors such as ungulate browsing and sapsucker stem wounding that trigger Cytospora infection are important drivers of willow decline (Kaczynski, 2013). The persistence of epicormic shoots that regenerate below the fungal infection is essential for the maintenance of a tall, intact riparian willow ecosystem, where stem mortality due to Cytospora canker occurs. Tall willows are needed to support beaver populations, whose dam building and flooding can mitigate the effects of future droughts and facilitate willow establishment, which is critical for long-term persistence of willow communities (Cooper et al., 2006).

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