

Altered leaf morphology, leaf resource dilution and defense chemistry induction in frost-defoliated aspen (*Populus tremuloides*)

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Received March 17, 2009; accepted June 18, 2009; published online August 10, 2009

Summary In May 2007, a widespread frost event defoliated much of Utah's high elevation aspen. About 5 weeks later, the frost-defoliated aspen produced a second leaf flush. The objective of this study was to characterize changes in leaf morphology and function in re-flush leaves following frost defoliation. Leaf size and thickness, photosynthesis, carbohydrate and nutrient status, and defense chemistry (phenolic glycosides and condensed tannins) were measured in first and second flush leaves. The second flush leaves produced two different morphological responses depending on frost damage severity. Severe frost damage was characterized by patchy canopy re-flushing with leaves that were on average four times larger than the first flush leaves. Moderate frost damage produced full canopy flushes with second flush leaves that were typically smaller than the first flush leaves. The second flush leaves tended to be thicker, and had significantly lower nutrient and sucrose concentrations, but had equal or higher rates of photosynthesis. These leaves showed a general pattern of defense chemistry induction with phenolic glycosides and condensed tannins increasing two- to threefold. Some of the changes in leaf morphology and defense chemistry observed in second flush leaves in 2007 persisted in leaves produced in the following year. We hypothesize that defense chemistry induction following abiotic defoliation serves as insurance against secondary defoliation events by herbivores that may further deplete nutrient and carbohydrate leaf resources below threshold points that are critical for physiological function. Resource dilution and allocation to secondary defense may place constraints on growth capacity.

Keywords: condensed tannins, defoliation, leaf flush, phenolic glycosides, temperature.

Introduction

Quaking aspen (*Populus tremuloides* Michx.) is the most widely distributed tree species in North America, and as a

result it experiences a wide variation in climatic conditions across its range. Its successful establishment across diverse landscapes demonstrates its resilience and adaptability as a species to environmental extremes. However, increases in aspen decline and dieback observed from western Canada (Hogg et al. 2002, Brandt et al. 2003) down through the central and southern Rocky Mountains (Rogers 2002, Worrall et al. 2008) suggest that current management strategies and changing climate conditions may impose constraints on aspen vigor in portions of its western range.

Climatic events such as drought and temperature extremes are important selection forces on biological organisms (McDowell et al. 2008), and can drastically alter plant community structure and ecosystem processes (Ciais et al. 2005, Holmgren et al. 2006). Extreme climatic events, which are projected to increase in North America under future climatic scenarios (Easterling et al. 2000, IPCC 2007), will likely have a profound influence on aspen communities, particularly on the margins of its range. While a substantial amount of work has been done examining the effects of drought events on aspen health (Frey et al. 2004), less is known about how temperature extremes influence aspen function and vigor. Evidence suggests that a widespread frost defoliation event in northern Arizona in the spring of 1999 was an inciting factor in subsequent aspen dieback and mortality (Fairweather et al. 2008). Insect defoliation events, which have been more thoroughly studied, can reduce carbon gain (Hart et al. 2000) and contribute to canopy dieback symptoms (Hogg et al. 2002).

Earlier and warmer spring conditions associated with climatic warming are projected to accelerate leaf phenology, leaving young foliage prone to late spring frost damage (Cannell and Smith 1986, Hanninen 2006). Early leaf flushing in response to periods of late winter warming followed by extreme low temperatures can produce extensive forest defoliation events in both sub-alpine and boreal forest systems (Korstian 1921, Cayford et al. 1959, Fairweather et al. 2008). In the two earlier studies, it was documented that frost damage occurred across a wide range of tree species,

but a consistent pattern emerged in which deciduous species tended to be more sensitive than conifer species, with aspen showing the greatest sensitivity (Korstian 1921, Cayford et al. 1959).

At extremely low temperatures, or during sensitive stages of development, frost can cause leaf necrosis leading to partial or complete canopy defoliation in aspen (Korstian 1921). After defoliation, aspen will often produce a second leaf flush several weeks later (Cayford et al. 1959). Severe frost events cause bud damage that results in patchy canopy re-flushing in the surviving leaf buds (Korstian 1921, Cayford et al. 1959). In this study, we also observed a moderate frost damage class in which all first flush leaves were lost, but bud damage was minimal resulting in full canopy re-flushing (S.B. St. Clair, personal observation). It is possible that the nutrient and carbon resources that are lost in the first leaf flush following defoliation will result in resource dilution in the second flush leaves, which may have adverse effects on leaf physiology. Low temperature has been shown to impair the photosynthetic function of aspen leaves that survive the frost events (Lamontagne et al. 1998), but little is known about how photosynthesis is altered in second flush leaves.

Aspen produces two classes of phenolic-based allelochemicals through the shikimic acid pathway: condensed tannins and phenolic glycosides. Leaf concentrations of these two secondary compounds vary significantly among aspen clones, but are generally found in high concentrations (Lindroth and Hwang 1996), representing a significant resource investment. Phenolic glycosides negatively affect insect herbivores, and greater foliar phenolic glycoside concentrations are correlated with lower levels of insect defoliation (Donaldson and Lindroth 2007). Condensed tannins are less important in deterring aspen adapted herbivores (Donaldson and Lindroth 2007) and their ecological significance in aspen remains unclear, although they can be induced in response to simulated defoliation (Osier and Lindroth 2004). A widespread defoliation event caused by an outbreak of forest tent caterpillars resulted in moderate decreases in tannins and a sixfold increase in phenolic glycosides in re-flushing leaves (Donaldson and Lindroth 2008). Following a combination of mechanical and insect defoliation, both phenolic glycoside and condensed tannin concentrations were increased in indeterminate shoots of aspen (Stevens and Lindroth 2005). It is, however, unclear whether insect elicitors associated with biotic defoliation events are required for defense chemistry induction, or if abiotic defoliations independent of herbivory are sufficient to elicit a defense response. A study conducted with hybrid poplar suggests that herbivory induces the strongest defense response (Havill and Raffa 1999).

The mountains of Utah experienced an extensive frost defoliation event in the spring of 2007. In response to early warm temperatures, aspen trees leafed out nearly a month early (Currit and St. Clair 2009). On 23 May the night-time temperature dropped to 6 °C, resulting in widespread frost

defoliation of Utah's high elevation deciduous trees. Using MODIS satellite images, we discovered that frost defoliation was most severe at mid-elevation in Utah's mountain belt with more damage occurring in the northern section of the state (Currit and St. Clair 2009). Site visits confirmed a widespread aspen defoliation in the Wasatch-Cache, Uinta and Fishlake National Forests, which span a significant latitudinal range in the state of Utah. About 4–5 weeks later, the frost defoliated aspen produced a second leaf flush, providing an opportunity to examine the potential changes in morphology and physiology of second flush leaves of frost-defoliated aspen. We tested the following hypotheses: (1) secondary leaf flushes have altered morphology; (2) second flush leaves are diluted in nutrient and carbohydrate resources relative to first flush leaves; (3) rates of photosynthesis and stomatal conductance are reduced in second flush leaves; and (4) secondary defense chemistry is induced in leaves produced subsequent to the frost defoliation event. We also examined the morphological, nutrient and defense chemistry traits in 2008 to determine whether leaf responses to the frost event persisted into the next growing season.

Materials and methods

Study sites

Two field experiments were conducted as part of this study. The first experiment was conducted at the Fishlake National Forest in the mountains of south central Utah, in the vicinity of South Last Chance (38°39'10.27" N and 111°30'13.87" W; elevation 2990 m). On June 21, 2007 we identified five aspen trees with an average stem diameter of 19 ± 1.8 cm that had been frost-defoliated on the west side of their canopy, but retained their leaves on the east side during the May 2007 frost period. Based on their proximity (within 10 m of each other), similar age (based on size) and morphological similarities, it is highly likely that these five trees were clonal. We took pictures of each of these five trees using a digital camera. On August 14, 2007, we returned to this field location where we observed that the frost-defoliated side of each of the five trees had produced a second leaf flush. Based on the pictures taken in June, we were able to identify branches that retained first flush leaves and branches that had produced second flush leaves in frost-defoliated branches. This allowed us to do a paired comparison of first and second flush leaves on the same tree. From each tree, we collected first and second flush leaves from low, mid and high canopy positions using a pole pruner. We immediately measured the rates of photosynthesis and stomatal conductance on two leaves of each branch segment we collected. We had measured stomatal responsiveness to branch break in aspen previously and found that the stomates begin to close on average 6 min after the branch is severed. Our measurements of gas

exchange typically take 60 s (see below), which is well before the stomates begin to close. We then stored the leaves in a cooler on ice for transporting back to the laboratory. Upon arrival at the laboratory, the leaves were frozen at -80°C until they were analyzed. Tissues analyzed in the laboratory were pooled samples of leaves collected from each of the three canopy positions for both first and second flush leaf samples. Data from each of these three positions was then averaged to get a mean value for first and second flush leaves of each tree. The following year, on August 13, 2008, we returned and re-sampled as in 2007, with the exception that we did not measure photosynthesis and stomatal conductance.

The second experiment was conducted along a 5-km transect at Deseret Ranch in the mountains of northeastern Utah ($41^{\circ}22'49.00''\text{N}$ and $111^{\circ}27'46.35''\text{W}$; elevation 2600 m), which spanned multiple clones based on morphological differences. Therefore, in the Fishlake study we eliminated genotypic contributions to variation in leaf traits (same clone) and in the Deseret Ranch study we were able to examine whether the defoliation effect was robust across a population of clones. The transect spanned a ridge line that experienced a significant frost defoliation during the May 2007 frost event. A trip to the location in June 2007 revealed a substantial frost defoliation, but also had trees that had retained their first flush leaves. We returned on August 3, 2007 to take measurements and to collect samples. Three different frost classes were identified (1) the control group that maintained their first flush leaves; (2) a moderate frost damage class in which the trees lost their first flush leaves, but undamaged leaf buds then produced a full secondary leaf flush throughout the canopy; and (3) a severe frost damage class, in which all first flush leaves were lost and frost damage of a large portion of the leaf buds resulted in patchy secondary leaf flushes from surviving leaf buds. Frost-defoliated trees in both moderate and severe damage classes could be identified because dead first flush aspen leaves persisted on the tree throughout the summer.

Within each damage class, eight aspen trees were selected for sampling. We selected aspen trees that were intermediate in age and consistent in size. Control, mild and severe frost damage groups had mean stem diameters (cm) and standard errors (SEs) of 23 ± 2.4 , 24 ± 2.2 and 21 ± 2.2 , which showed no statistical difference among the treatment classes. Replicate trees within each damage class were selected at about 0.5-km intervals along the transect. A branch of leaves from a low, intermediate and high position in the canopy were collected from each tree using a pole pruner. Immediately upon collection, we measured the rates of photosynthesis and stomatal conductance (see description below) on two separate leaves of each branch segment we collected. We then placed the leaves in labeled freezer bags in a cooler on ice for transporting back to the laboratory. Upon arrival at the laboratory, the leaves were frozen at -80°C until further analysis was conducted. Tissues analyzed in the laboratory represented a pooling

of leaves collected from each of these three canopy positions. Data from each of these three positions was then averaged to get a single data value for each tree.

The following year, on July 31, 2008, we returned to Deseret Ranch and re-sampled as in 2007, with the exception that we did not repeat the measurements of photosynthesis and stomatal conductance, and the samples were collected only from control and severe frost damage classes. In both 2007 and 2008, we made visual estimates of the percent leaf loss of each of the trees in the severe frost damage class. Temperature data for the spring 2007 period was measured in the vicinity of the Deseret Ranch transect at the Lightning Ridge meteorological station, which is part of the Utah Snotel network (<http://www.wcc.nrcs.usda.gov/snotel/snotel.pl?sitenum=1056&state=ut>).

Leaf gas exchange

Leaf gas exchange measurements were made on the two youngest fully expanded leaves of the branch segment. The rates of photosynthesis (A_{max}) and stomatal conductance (g_s) were measured using a gas exchange system (LI-COR 6400, LI-COR Environmental Inc., Lincoln, NE). Gas exchange was measured at a photosynthetic photon flux density of $2000\ \mu\text{mol m}^{-2}\text{s}^{-1}$ generated by a blue-red LED light source at ambient temperature and humidity. Baseline leaf and reference chamber CO_2 concentrations of $375\ \mu\text{mol mol}^{-1}$ were achieved using a CO_2 mixer. Measurements were initiated by sealing the leaf in the chamber. When CO_2 and water vapor concentrations in the leaf chamber reached a steady state (60–90 s), the rates of photosynthesis and stomatal conductance were logged. All measurements were taken between 10:00 and 15:00 h.

Leaf morphological traits

In the laboratory, the frozen leaf samples were quickly run through a leaf area meter (LI-COR 3000, LI-COR Environmental Inc., Lincoln, NE) to determine the area of each leaf sample. Care was taken to prevent the leaf samples from sitting out at room temperature and thawing. The leaves were then freeze dried for 48 h to preserve the chemical integrity of the tissue (Lindroth and Koss 1996). Finally, the leaf samples were measured for dry mass using an analytical balance (GeneMate GP-600, ISC Bioexpress, Kaysville, UT). Specific leaf area (SLA) was calculated based on the square centimeter of leaf area per gram dry weight of leaf tissue.

Carbohydrate analysis

Freeze-dried leaf samples were ground and homogenized using a Wiley Mini-Mill (Thomas Scientific, Swedesboro, NJ). Glucose and sucrose were extracted from leaf tissue according to the methods of Hendrix (1993). About 20 mg of freeze-dried leaf sample was placed in screw-capped micro-centrifuge tubes and then suspended in

0.67 ml of 80% ethanol. The samples were then extracted by placing them in a water bath at 80 °C for 20 min. The supernatant was removed and placed in a separate 2-ml test tube. The ethanol extraction was repeated two more times for a final extract volume of 2 ml. We placed 20 µl of sample extracts in micro-plate wells and placed them in a drying oven for 20 min at 55 °C to evaporate off the ethanol. The dried samples were then re-suspended in 20 µl of water.

Standard curves were prepared using pure glucose and sucrose standards. One hundred microliters of the glucose reaction mix (GOPOD, Megazyme, Wicklow, Ireland) was then added to the sample and the standard wells. The micro-plates were capped and incubated for 20 min at 50 °C in a water bath. Absorbance was read at 510 nm using a spectrophotometer (SpectraMax Plus 384, MDS, Toronto, Canada). Invertase (Sigma, St. Louis, MO) in a 10-µl volume was then added to the sample and sucrose standard wells. The samples were incubated in the dark at 37 °C for 15 min. Absorbance was read again at 510 nm in the spectrophotometer. Sucrose concentration was determined according to the increase in absorbance following the addition of invertase.

Starch was isolated from the ethanol-extracted tissue sample in 2 ml of water in a screw-capped tube that was autoclaved for 1 h at 135 °C. We placed 50 µl of extracted samples in wells within a 96-well plate. A standard curve was prepared using a starch standard. We added 50 µl of starch assay reagent (Starch HK Assay Kit-SA20, Sigma, St. Louis, MO) to the sample and the standard wells. The plate was capped and incubated with shaking at 60 °C for 15 min. The plate was cooled to room temperature, and 200 µl of glucose assay reagent (Starch HK Assay Kit, Sigma, St. Louis, MO) was added to each well. Absorbance was then read at 340 nm using the spectrophotometer.

Secondary chemistry

Phenolic glycosides were extracted from 50 mg of freeze-dried leaf tissue in 1 ml of methanol. The samples were vortexed at high speed for 5 min. The liquid supernatant was then removed and placed in separate micro-centrifuge tubes. This procedure was repeated for a final 2-ml volume of sample extract. Phenolic glycoside concentrations (salicortin and tremulacin) were quantified using high-performance liquid chromatography (Agilent 1100 Series, Santa Clara, CA) with a Luna 2, C18 column (150 × 4.6 mm, 5 µm) at a flow rate of 1 ml min⁻¹. Compound peaks were detected using a UV lamp at a wavelength of 280 nm using purified salicortin and tremulacin standards isolated from aspen leaves (Lindroth et al. 1993).

Condensed tannins were extracted from 50 mg of freeze-dried leaf tissue. The extraction consisted of placing the leaf material in a screw-capped micro-centrifuge tube and suspending the material in 1 ml of a 70% acetone–10 mM ascorbic acid solution. The sample was then vortexed at high speed for 20 min at 4 °C. The liquid supernatant

was removed and placed in a separate micro-centrifuge tube, and the extraction was then repeated. Condensed tannin concentrations were measured with a spectrophotometer (SpectraMax Plus 384, MDS, Toronto, Canada) using the acid butanol method (Porter et al. 1986) with purified condensed tannins standard isolated from aspen leaves (Hagerman and Butler 1980).

Element analysis

In preparation for elemental analysis, 250 mg of dry leaf tissue was wet digested in 5 ml of concentrated HNO₃ for 12 h at room temperature and then for an additional 10 min at 104 °C. We added 1 ml of perchloric acid to the sample, which continued to boil at 104 °C for an additional 60 min. Sample volume was then brought to a final 50-ml volume by deionized water. The leaf concentrations of P, S, K, Ca, Mg and Fe were determined using inductively coupled plasma spectroscopy (Iris Intrepid II XSP, Thermo Electron Cooperation, Waltham, MA) (Dahlquist and Knoll 1978). For the determination of leaf nitrogen, 50 mg of dry leaf tissue was placed in a tin capsule and analyzed in a nitrogen analyzer (TruSpec, CN Determinator, LECO Cooperation, St. Joseph, MI) using the combustion method (Campbell 1991).

Statistical analysis

A matched-pair analysis was performed using a paired *t* test to compare the difference in response means of first and second flush leaves from the Fishlake study. A one-way analysis of variance (ANOVA) was used to examine the effects of frost severity on each of the response variables measured in the Deseret Ranch transect study in 2007. Repeated-measures ANOVA was used to test the effects of frost defoliation on response variables measured over both years (2007 and 2008) using time as the 'within' factor (Gumpertz and Brownie 1993). Mean comparisons among treatment groups were determined using a Student's *t* test. *P* values were determined from type III sum of squares estimates, and statistical significance was defined as $\alpha \leq 0.05$. Dependent variables were tested for normality and homogeneity of variance using Shapiro–Wilk *W* statistics and equal variance tests. Data met the assumptions of normality with the exception of the 2007 leaf area and starch data from Deseret Ranch, which we transformed using a Box–Cox transformation. A Welch ANOVA test was used in the rare case when the data did not meet the requirements of homogeneity of variance. Statistical analyses were performed using JMP Version 7 statistical software (SAS Institute, Cary, NC).

Results

Temperature

Minimum, high and mean daily temperatures were unusually warm in the vicinity of the Deseret Ranch transect site

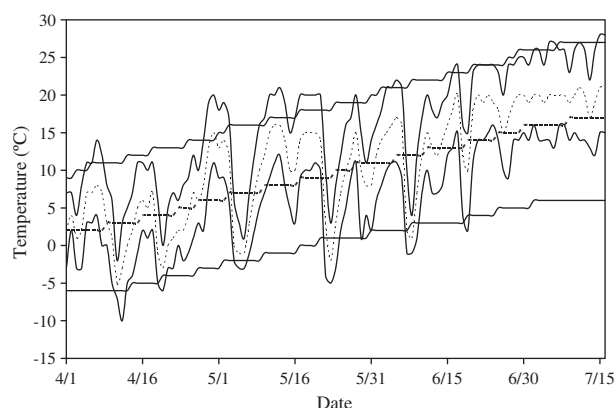


Figure 1. Air temperature data from Lightning Ridge in the vicinity of the Deseret Ranch transect study from April 1 to July 15, 2007 relative to the long-term temperature averages (1971–2000). Upper and lower solid lines represent daily average maximum and minimum temperatures with the dotted line representing the daily mean. Graph by Andreas Leidolf.

in parts of April and early May of 2007 (Figure 1). This warm period was followed by three periods of abnormally low freezing temperatures in late May and in early June (Figure 1), with most of the frost damage likely occurring on May 23, 2007.

Canopy re-flushing and leaf morphology

Visual estimates of trees in the severe frost damage class at Deseret Ranch showed a 55% loss of first flush leaves that did not re-flush because of frost damage to the leaf buds. Visual assessments in the following year showed that the leaf loss was permanent as the severely frost-defoliated branches of 2007 did not produce leaves in 2008.

In both years of the Fishlake study, leaves produced in frost-defoliated portions of the canopy were on average only half as large as leaves in undamaged portions of the canopy (Table 1). In contrast, SLA was not affected by the frost damage in either year of the study (Table 1). Control and frost affected leaves collected in 2008 were on average only half as big as leaves in 2007 (Table 1). Second flush

leaves produced in severe frost-damaged aspen in the Deseret transect experiment were 3–4 times larger than control or the moderate frost damage leaves, a result that was consistent in both 2007 and 2008 (Figure 2; Table 2). Both frost damage classes tended to have thicker second flush leaves than first flush control leaves as indicated by their lower SLA values (Figure 2). In comparing control and severe frost damage classes, this response was consistent across both years, although SLA decreased significantly from 2007 to 2008 (Figure 2; Table 2).

Leaf gas exchange

There were no statistical differences in the rates of photosynthesis in first and second flush leaves in the Fishlake study in the summer of 2007 (Table 1). In the Deseret transect study, second flush leaves of both frost damage classes had significantly higher rates of photosynthesis and stomatal conductance than control leaves in August 2007 (Figure 3).

Non-structural carbohydrates

Leaf sucrose concentrations in second flush leaves were significantly lower than those in first flush leaves in both studies (Table 1; Figure 4). Second flush leaves of the moderate frost damage class in the Deseret study also had significantly lower glucose concentrations, while all other differences for glucose were not significant. Foliar starch concentrations showed no significant differences among the frost classes in either study.

Nutrients

In the Fishlake study, a clear pattern emerged in which second flush leaves had significant reductions in all foliar nutrients we analyzed, except Mg (Table 3). Nitrogen concentrations were reduced by 24% (3.04–2.31%) in second flush leaves. The difference in foliar N was still statistically significant ($P = 0.002$) in leaves collected from the same branches in 2008, but the difference had narrowed to 12% (1.88–1.60%).

Table 1. Differences in leaf morphology, defense chemistry, photosynthesis (A_{\max}) and non-structural carbohydrates in paired samples collected from aspen trees in the Fishlake study. Mean values ($n = 5$) presented with SE. A matched pair's analysis was run to test for differences between first and second leaf flush leaves in 2007. Leaf morphology and defense chemistry traits are also presented for 2008. Statistical significance designated as $P \leq 0.05$.

	Leaf area (cm ²)	SLA (cm ² g ⁻¹)	Phen. Glyc. (% dry wt.)	Tannins (% dry wt.)	A_{\max} ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	Glucose (% dry wt.)	Sucrose (% dry wt.)	Starch (% dry wt.)
2007								
First flush	13.6 \pm 1.8	112 \pm 3.8	5.0 \pm 0.7	11.0 \pm 2.7	12.4 \pm 0.7	2.9 \pm 0.01	4.0 \pm 0.2	9.1 \pm 0.5
Second flush	6.9 \pm 0.3	114 \pm 6.3	8.5 \pm 0.9	20.2 \pm 1.2	12.9 \pm 0.4	3.6 \pm 0.3	3.3 \pm 0.2	9.4 \pm 0.3
<i>P</i> value	< 0.0001	0.577	0.003	0.030	0.417	0.116	0.054	0.675
2008								
First flush	6.0 \pm 0.4	111 \pm 3.9	5.3 \pm 0.6	2.4 \pm 0.2				
Second flush	3.3 \pm 0.2	121 \pm 2.0	4.9 \pm 1.3	6.2 \pm 0.4				
<i>P</i> value	0.009	0.093	0.759	0.002				

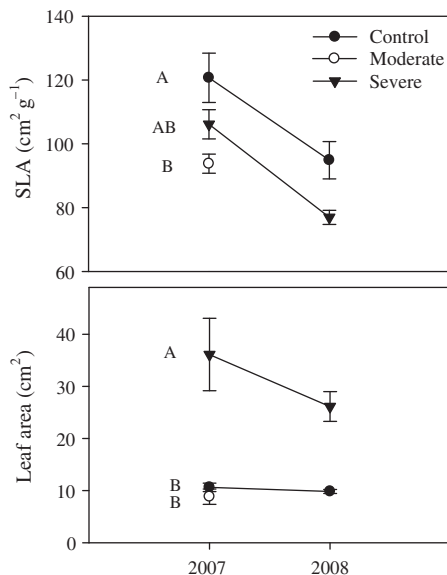


Figure 2. Symbols represent mean differences in leaf area and SLA in leaf samples collected in the Deseret Ranch transect study in 2007 and 2008. Mean values ($n = 8$) presented with SE bars. Different letters indicate statistically significant differences based on a Student's t test with significance designated as $P \leq 0.05$.

Table 2. F values from the repeated-measures ANOVA model testing the effects of the frost and time (2007 versus 2008) and their interaction on leaf morphology, defense chemistry and N concentration in the Deseret transect study. Significance designated as * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

	Leaf area	SLA	Phen. Glyc.	Tannins	N
Frost	19.1**	6.63*	30.1***	1.68	0.081
Time	1.07	22.9**	15.8**	7.24*	7.85*
$F \times T$	0.70	0.01	0.04	1.07	1.66

In the Deseret study, second flush leaves of severe frost-defoliated aspen trees had significantly lower concentrations of foliar N and Fe than control or second flush leaves of trees that experienced moderate frost damage (Table 4). All other statistically significant differences were the result of second flush leaves from one or both frost damage classes having higher base cation (Ca and Mg) concentrations than control leaves (Table 4).

Leaf defense chemistry

In the Fishlake study, phenolic glycosides and condensed tannins were induced in second flush leaves in 2007 (Table 1). In 2008, condensed tannins remained significantly higher in leaves produced on frost-defoliated branches. In contrast, the significant increase in phenolic glycoside concentrations of second flush leaves observed in 2007 did not persist into 2008 (Table 1). While phenolic glycoside concentrations in first flush leaves remained

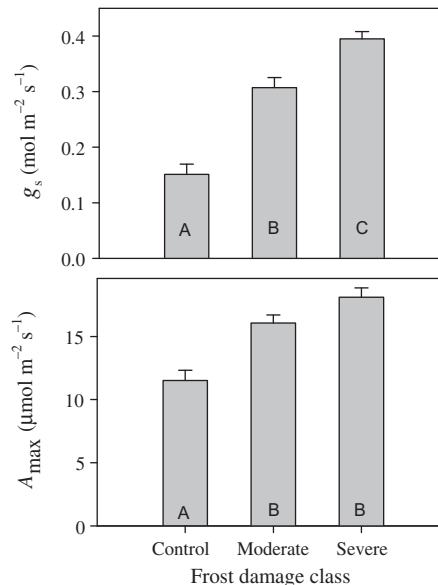


Figure 3. Mean differences in photosynthesis (A_{max}) and stomatal conductance (g_s) of leaves within each of the three frost damage classes in the Deseret Ranch transect study in 2007. Mean values ($n = 8$) presented with SE bars. Different letters indicate statistically significant differences based on a Student's t test with significance designated as $P \leq 0.05$.

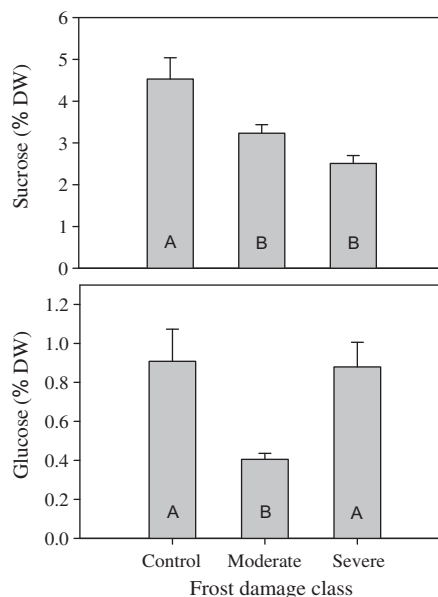


Figure 4. Mean differences in glucose and sucrose concentrations of leaves from trees within each of the three frost damage classes in the Deseret Ranch transect study in 2007. Mean values ($n = 8$) presented with SE bars. Different letters indicate statistically significant differences based on a Student's t test with significance designated as $P \leq 0.05$.

constant from 2007 to 2008, phenolic glycoside concentrations in second flush leaves and condensed tannins in both leaves from control and frost-defoliated branches were reduced from 2007 to 2008 (Table 1).

Table 3. Foliar chemistry of paired samples collected from aspen trees in the Fishlake study. Mean values ($n = 5$) presented with SE. A matched pair's analysis was run to test for differences between first and second leaf flushes produced in the 2007 field season. Statistical significance designated as $P \leq 0.05$.

2007	N (%)	P (mg g ⁻¹)	K (mg g ⁻¹)	Ca (mg g ⁻¹)	Mg (mg g ⁻¹)	Fe (mg g ⁻¹)
First flush	3.04 ± 0.1	2.37 ± 0.1	14.5 ± 0.9	14.9 ± 1.3	2.6 ± 0.07	1.4 ± 0.01
Second flush	2.31 ± 0.06	1.94 ± 0.05	13.5 ± 0.8	10.3 ± 0.5	2.6 ± 0.1	1.0 ± 0.01
<i>P</i> value	0.016	0.066	0.036	0.035	0.841	0.020

Table 4. Foliar chemistry comparing mean differences among the three frost treatment groups in the Deseret Ranch transect study. Mean values ($n = 8$) presented with SE. A one-way ANOVA was used to test for differences between first and second leaf flushes in the 2007 field season. Different superscript letters indicate statistically significant differences based on a Student's *t* test. Statistical significance designated as $P \leq 0.05$.

2007	N (%)	P (mg g ⁻¹)	K (mg g ⁻¹)	Ca (mg g ⁻¹)	Mg (mg g ⁻¹)	Fe (mg g ⁻¹)
First flush (control)	2.12 ± 0.1 ^a	1.63 ± 0.1 ^a	10.3 ± 1.2 ^a	12.1 ± 1.0 ^a	1.85 ± 0.2 ^a	0.28 ± 0.03 ^a
Second flush (moderate)	2.08 ± 0.1 ^a	2.06 ± 0.1 ^a	12.9 ± 1.1 ^a	15.4 ± 1.0 ^b	2.44 ± 0.2 ^{ab}	0.26 ± 0.03 ^a
Second flush (severe)	1.77 ± 0.1 ^b	1.97 ± 0.1 ^a	11.7 ± 1.2 ^a	11.4 ± 1.1 ^a	2.63 ± 0.2 ^b	0.17 ± 0.03 ^b
<i>P</i> value	0.039	0.128	0.336	0.031	0.040	0.063

In the Deseret study, second flush leaves in the moderate frost damage class had significantly higher condensed tannin concentrations than leaves in the control or severe damage groups (Figure 5; Table 2). There were no significant differences in condensed tannins in 2007 or 2008 between the control and the severe frost damage class (Figure 5; Table 2). The significant time variable in the repeated measures model indicates a consistent reduction in tannins from 2007 to 2008 (Table 2). In contrast, severely frost-defoliated aspen tree leaves had significantly higher phenolic glycoside concentrations in both 2007 and 2008 than control leaves (Figure 5; Table 2). The significant time variable in the repeated-measures model indicates a substantial increase in leaf phenolic glycoside concentrations in both control and severe frost damage trees from 2007 to 2008 (Figure 5).

Discussion

Although a significant amount of research has explored how trees are affected by insect defoliation, much less is known about the response of trees to abiotic defoliation. Insect defoliation events are episodic in nature (Fitzgerald 1995, Liebhold et al. 2000) and so are frost defoliation events, although the frequency of the latter is poorly characterized. Although climate warming has and is projected to continue increasing the number of frost-free days (Easterling 2002), mild temperature spells in winter and in early spring are expected to accelerate phenology and growth onset, which will make trees in boreal and temperate zones more prone to late spring frost damage (Hanninen 2006). Our temperature data (Figure 1) and image analysis (Currit and St. Clair 2009) are consistent with this prediction, as we observed that early spring

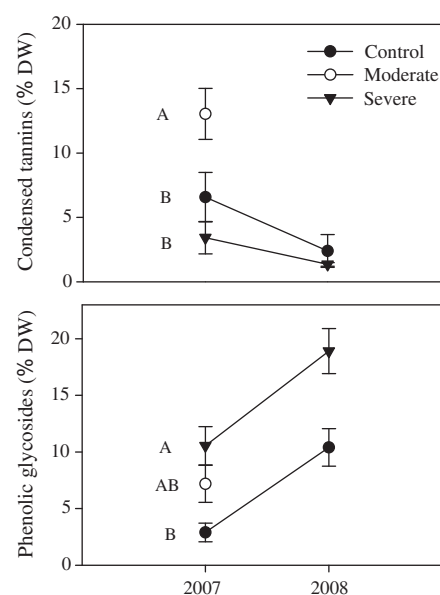


Figure 5. Symbols represent mean differences in defense chemistry in leaf samples collected in the Deseret Ranch transect study in 2007 and 2008. Mean values ($n = 8$) presented with SE bars. Different letters indicate statistically significant differences based on a Student's *t* test with significance designated as $P \leq 0.05$.

warming in 2007 accelerated leaf flushing, which in response to subsequent below freezing temperatures resulted in a widespread frost defoliation. Although temperature conditions have a direct role in frost damage, sensitivity to frost defoliation can vary significantly among adjacent clones (S.B. St. Clair, personal observation). Variation in leaf phenology in the spring, which can vary by several weeks among aspen clones (Donaldson and

Lindroth 2008), is likely an important trait controlling frost sensitivity in aspen.

The results from this study supported our first hypothesis, and are consistent with those from other studies that have shown altered leaf morphology of second flush aspen leaves following frost defoliation (Korstian 1921, Cayford et al. 1959). What causes the production of abnormally large aspen leaves in response to frost damage has not been extensively explored. There is no evidence that insect defoliations cause such a dramatic change in the morphology of second flush leaves. In contrast, we typically observed smaller leaves in trees that experienced more moderate frost damage when there was little to no bud loss. Since a significant portion of the buds were damaged in the severe frost trees, it is reasonable to think that the production of large leaves is related to bud mortality. We hypothesize that resources reserved for a full secondary canopy re-flushing may be directed into the surviving growing points resulting in the production of large leaves. Alternatively, the response may be related in some way to changes in hormones produced by buds, which are known to control resource allocation in aspen (Wan et al. 2006). The reduction in leaf area in response to moderate frost damage in the Fishlake study may again be related to the influences on bud meristem activity, or stored resources may have been insufficient to produce normal-sized leaves throughout the entire canopy. Leaf thickening in second flush leaves of both frost damage classes in the Deseret study is intriguing as it persisted into the second year and because changes in SLA have important impacts on other leaf functional traits (Wright et al. 2004).

Generally, our second hypothesis regarding nutrient and carbohydrate reduction in second flush leaves was also supported by the data. Stevens and Lindroth (2005), following a combination of mechanical and insect defoliation, did not observe a reduction in foliar N in indeterminate re-growth of leaves in second year aspen ramets. Following complete forest tent caterpillar defoliation in more mature aspen trees, a 30% reduction in foliar N of second flush leaves was observed (Donaldson and Lindroth 2008). The Fishlake study shows that the dilution effect is a general pattern across the major leaf nutrients. Patterns of foliar N and Fe accumulation from the Deseret study corroborate this pattern. Since N is likely the nutrient that most limits aspen productivity across its range, its reduction has the greatest potential to affect physiological function and growth (Van Cleve and Oliver 1982).

An interesting finding in our study was that reductions in foliar N concentrations persisted into the second growing season in leaves produced on frost-defoliated branches in the spring of 2007. Donaldson and Lindroth (2008) showed that the foliar N concentrations remained 30% lower than the pre-defoliation levels 2 years after insect defoliation (Donaldson and Lindroth 2008). Defoliation of aspen has been shown to reduce xylem production, which transports both water and nutrients from the soil (Jones et al. 2004).

A loss of xylem production is one possible explanation for the maintenance of reduced foliar N concentrations in defoliated aspen in subsequent growing seasons.

Our results indicate that although foliar nutrient concentrations were reduced in second flush leaves, they did not drop below deficiency thresholds for optimal photosynthetic function. Contrary to our third hypothesis, photosynthesis was stimulated in second flush leaves in the Deseret study. This could partially be the result of thickening (reduction in SLA) in the second flush leaves. Thicker leaves have been shown to have higher rates of photosynthesis because they have more photosynthetic machinery (pigment and enzymes) per unit leaf area (Sefton et al. 2002). It appears that increased stomatal conductance in re-flushing leaves also contributed to increased rates of carbon fixation. However, increases in photosynthesis would only partially offset carbon losses associated with leaf mortality and leaf area loss. In this study, both temporary and longer-term canopy leaf losses occurred. First, there was a 5–6-week period after the frost event and before re-flushing in which there was no leaf tissue present. Second, reduction in leaf size (intermediate frost class) or permanent leaf loss (severe frost class) reduced the whole canopy leaf area in 2007, which was a pattern that persisted into 2008. Although individuals in the severe frost damage class survived, follow-up studies are needed to determine whether tree function can be maintained with such large and permanent losses in leaf area over time. This may partially explain the association between a major frost defoliation event in 1999 and a subsequent aspen dieback in northern Arizona (Fairweather et al. 2008). Since leaves are a primary site of auxin synthesis that maintains apical dominance in aspen, it is possible that permanent leaf loss may shift the hormone balance and trigger root suckering as a mechanism of survival following severe frost damage (Wan et al. 2006).

This is the first study that we are aware of that demonstrates defense chemistry induction in second flush leaves following abiotic defoliation, a trend that is consistent with our fourth hypothesis. Osier and Lindroth (2004) showed induction of tannins in first flush leaves in response to artificial defoliation using scissors, but no significant changes in phenolic glycosides. The two- to threefold induction of defense chemistry in second flush leaves illustrates that aspen defoliation can elicit a strong defense response independent of herbivore elicitors. The level of induction is also significant because it increases phenolic glycoside concentrations to a range that is substantially more effective in deterring insect defoliation (Donaldson and Lindroth 2007). However, it also represents a significant resource investment and raises the question of its adaptive value. One possibility is that defoliation functions as a primary cue for insect herbivory and in the case of an abiotic defoliation, the aspen incorrectly responds to frost defoliation as an insect attack. Alternatively, defense chemistry induction may be beneficial by serving as an ‘insurance policy’ against a secondary defoliation event by insects (Donaldson

and Lindroth 2008), pathogens (Buzzini et al. 2008) or ungulate browsers (for younger ramets) (Wooley et al. 2008) that would further deplete nutrient resources below the threshold levels that would severely constrain leaf physiological function.

Second flush leaf samples collected for this study in 2007 were between 3 and 5 weeks younger than first flush leaves that served as a comparison. We recognize that frost damage effects on first and second flush leaves could be confounded by the differences in leaf age. However, evidence from the literature suggests that phenolic glycoside and condensed tannin concentrations in aspen leaves stabilize about 5 weeks after leaf-out (Roth et al. 1998, Osier et al. 2000) and remain relatively constant through the end of summer (Lindroth et al. 2002, Stevens and Lindroth 2005). Since the leaves were collected in August, this would have given first and second flush leaves ample time to reach stable levels of tannins and phenolic glycosides well ahead of senescence. Additionally, leaf samples collected in 2008 were of the same age developmentally and yet maintained a similar pattern of defense chemistry induction that was observed in 2007, providing more evidence that frost defoliation, not differences in leaf age, was the primary factor behind the observed differences in defense chemistry.

In the upper Midwest of the US into Canada, aspen experiences regular and expansive outbreaks by defoliating insects (Mattson et al. 1991). In the western US, widespread defoliation events tend to be less common, but several species can be important defoliators of aspen (Jones et al. 1985). A recent analysis suggests that projected warming trends in western North America are likely to promote the establishment of gypsy moth populations in the western US (Logan et al. 2007) and increase the range and density of other insect herbivores in the sub-alpine and boreal forests where aspen is common (Ayres and Lombardero 2000). This along with the evidence that warming trends will increase the frequency of frost defoliation events (Hanninen 2006) suggests that aspen may experience defoliation more frequently under future climate scenarios. Our results suggest that increases in defoliation frequency will impact aspen canopy and leaf morphology, reduce foliar nutrient concentrations and increase carbon allocation to defense chemistry production with a net negative effect on aspen vigor and health.

Acknowledgments

Special thanks to Bob Campbell for sharing his insights of western aspen ecosystems and for encouraging our research on the Fishlake National Forest. We are grateful to Rick Lindroth for providing well-tested laboratory protocols for conducting the defense chemistry analysis. Finally, thanks to Andreas Leidolf for designing Figure 1, Ron Ryel for insightful discussion of the frost damage at Deseret Ranch and Rick Danvir for facilitating our work at Deseret Ranch.

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