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SHORT COMMUNICATION

In situ Effects of Elevated Atmospheric CO_2 on Leaf Freezing Resistance and Carbohydrates in a Native Temperate Grassland

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The objectives of this study were to quantify changes in leaf freezing resistance and carbohydrate concentrations caused by long-term (6 years) exposure to elevated CO_2 (ambient: 360 µl l^{-1}, elevated: 600 µl l^{-1}) in five dominant plant species growing *in situ* in a native temperate grassland. Across all five species tested from three functional groups, the mean temperature at which all leaves were damaged (T_{100}) significantly ($P = 0{\cdot}016$) increased from −9·6 to −8·5 °C under elevated CO_2, and a similar marginally significant ($P = 0{\cdot}079$) reduction was observed for the mean temperature that caused 50 % leaf damage (T_{50}), from −6·7 to −6·0 °C. The mean temperature at which initial leaf damage was observed (T_0) was not significantly influenced by elevated CO_2. Although concentrations of soluble sugars (+25 %, $P = 0{\cdot}042$), starch (+53 %, $P < 0{\cdot}001$), and total non-structural carbohydrates (TNC, +40 %, $P < 0{\cdot}001$) were significantly higher under elevated CO_2, leaf freezing resistance actually *decreased* under elevated CO_2. Concentrations of soluble sugars were positively correlated with freezing resistance when viewed across all five community dominants, but within any individual species, no such relationships were found. We also found no evidence for our original hypothesis that increased concentrations of soluble sugars increase freezing resistance. Thus, future atmospheric CO_2 levels may instead increase the risk of late spring freezing damage. Furthermore, the strong differences in freezing resistance observed among the species, along with decreased freezing resistance, may increase the risk of losing species that have inherently weak freezing resistances from the plant community.

Key words: CO_2 enrichment, frost hardiness, sugar, starch, total non-structural carbohydrates (TNC).

INTRODUCTION

One almost universal response of plants to exposure to elevated CO_2 is an increase in the concentration of total non-structural carbohydrates (TNC; starch plus soluble sugars) in leaves (e.g. Wong, 1990; Farrar and Williams, 1991; Bazzaz and Fajer, 1992; Körner and Arnone, 1992; Körner and Miglietta, 1994; Poorter *et al.*, 1997). It has often been hypothesized that these increases in leaf carbohydrate concentrations may increase leaf freezing resistance. Evidence that carbohydrates confer freezing resistance is provided by the positive correlations that have been observed under ambient CO_2 between soluble carbohydrate concentrations and freezing resistance in a number of plant species (e.g. Sakai and Yoshida, 1968; Levitt, 1972; Kaurin *et al.*, 1981; Alberdi *et al.*, 1989; Vágújfalvi, 1999), as well as the observation that sugars can protect sensitive membranes such as thylakoids against freezing damage (Heber and Santarius, 1973). It is also believed that during frost hardening starch is hydrolysed to soluble, low-molecular weight sugars (Levitt, 1972). Thus higher starch concentrations under elevated CO_2 may also enhance freezing resistance in leaves.

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However, the only study to measure both leaf freezing resistance and carbohydrate concentrations of plants grown under elevated atmospheric CO_2 found no CO_2 effects on either leaf freezing resistance or carbohydrate concentrations and did not examine the relationship between the two (Wiemken *et al.*, 1996). Repo *et al.* (1996) and Lutze *et al.* (1998) have also quantified the effects of elevated CO_2 on leaf freezing resistance, but did not present leaf carbohydrate data. Lutze *et al.* (1998) found a decreased freezing resistance in leaves of field-grown *Eucalyptus* under elevated CO_2. They found that 31 % more leaves were damaged under elevated CO_2 than under ambient CO_2, and that this was consistent with the 1·4 K higher ice nucleation temperature they measured in leaves produced under elevated CO_2. In contrast, Repo *et al.* (1996) reported increased frost hardiness in pine needles in the first year of exposure to elevated CO_2, but found that this effect disappeared in the second growing season. Data from several other studies suggest that elevated CO_2 may alter freezing resistance of plants by altering plant phenology (Margolis and Vézina, 1990; Murray *et al.*, 1994; Wayne *et al.*, 1998). However, all of the above-mentioned studies were conducted with a single tree species in the seedling or sapling stage, and used plants growing in containers or in planted systems. The effects of elevated CO_2 on leaf freezing

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resistance of grassland species and the relationship with leaf carbohydrate chemistry have not been addressed.

The objectives of this study were to (1) characterize the effects of long-term (6 years) exposure to elevated CO_2 on leaf freezing resistance early in the growing season in a native temperate grassland when the risk of tissue damage due to late frost events is especially high, and (2) test whether changes in leaf freezing resistance are related to changes in leaf sugar, starch, or overall TNC concentrations. We hypothesized that freezing resistance would increase under elevated CO_2 due to higher leaf carbohydrate concentrations. We included five of the most abundant plant species in this grassland, representing three different functional groups, to assess species-specific changes in leaf freezing resistance, and to infer how these changes might translate into shifts in plant community species composition.

MATERIALS AND METHODS

Study site and experimental design

The study site is located in a nutrient-poor calcareous grassland in NW Switzerland (47°33′N 7°34′E, 520 m). The area was extensively grazed by cattle before the experiment began in 1993, after which mowing replaced grazing (Leadley *et al*., 1999). This management had no apparent effect on plant species composition (Schläpfer *et al*., 1998). The grassland consists of over 70 vascular plant species (Huovinen-Hufschmidt and Körner, 1998), and is dominated by the perennial grass *Bromus erectus* Hudson, which represents approx. 43 % of the above-ground biomass. Due to low summer precipitation, southwest exposure, and a shallow soil profile, the grassland regularly experiences drought during summer.

From March 1993, eight 1·2 m^2 experimental plots of natural calcareous grassland were maintained at current ambient CO_2 levels, and eight at elevated CO_2 (600 µl l^{-1}) using a screen-aided CO_2 control technique (Leadley *et al*., 1997). Each of the hexagonal plastic screens was 50 cm tall, which roughly corresponds to the height of the canopy at peak season. A 7 cm gap between the bottom of the chamber and the ground ameliorated some of the effects that open-top chambers have on climate, provided better turbulent mixing of CO_2, and allowed small herbivores (snails, slugs) to move freely into the chambers. CO_2 concentrations were controlled in each chamber independently. For a more detailed description of the study site, the experimental design and the CO_2 control technology see Leadley *et al*. (1997).

Field sampling

To quantify leaf freezing resistance, 30 leaves from five different species were sampled in each of the 16 experimental plots in the sixth growing season (May 1998). The five species were chosen because they are the most abundant species in this grassland and belong to three different functional groups; one graminoid species (*Bromus erectus* Hudson, Poaceae), three non-legume forbs (*Cirsium acaule* Scopoli, Asteraceae, *Sanguisorba minor* L., Rosaceae, *Salvia pratensis* L., Lamiaceae) and one legume forb (*Trifolium medium* L., Fabaceae). Because the freezing tests required many leaves, leaves of two adjacent experimental plots were pooled to ensure a sufficient number of leaves for calculating freezing damage in each species, resulting in four samples of 60 leaves per CO_2 treatment and species (experimental unit $n = 4$). Leaves were sampled in the early morning (between 0500 and 0700 h) because temperatures are lowest at this time of the day and freezing resistance is most critical. After clipping, leaves used to test freezing resistance were immediately placed in a dark insulated box and kept at 5 °C. Freezing tests began within 4 h of clipping.

At the same time, an additional set of leaves was clipped for the determination of carbohydrates. These leaves were dried immediately after harvest (within 5 min) in a microwave oven (2 min, 850 W). We placed a beaker with 200 ml of water in the microwave to absorb excess energy and prevent overheating of the samples (Körner and Miglietta, 1994; Popp *et al*., 1996). Final drying was completed in the laboratory using a convection drying oven at 60 °C for 24 h.

Determination of leaf freezing resistance

To estimate leaf freezing resistance, the 60 leaves from each species and experimental unit were packed in aluminum foil in sets of ten leaves (i.e. six packs of ten leaves per species and experimental unit) to achieve a good distribution of temperature and to prevent drying of the leaves during freezing tests. One of the six packs was exposed to one of five different target temperatures, while a control pack was maintained at 5 °C. Exposure to the five freezing temperatures was accomplished using five polystyrene isolated cylindrical aluminum chambers with a radius of 4 cm and depth of 7 cm. Temperature in each chamber was regulated separately by a computer-controlled Peltier cooling element. Temperature was reduced linearly in all chambers at equal speed from 5 to 0 °C. The rate of cooling below 0 °C differed for each chamber to ensure that target temperatures for all chambers were reached simultaneously in 2 h. The highest ('warmest') of the five target temperatures was determined for each species separately in preliminary tests with control leaves, and was set as the lowest temperature at which no damage under both ambient and elevated CO_2 concentrations occurred. This temperature was followed by four successively lower temperatures in 2 K steps. Target temperatures were maintained for another 2 h, long enough to ensure maximum possible damage (preliminary tests), before the temperature was linearly increased back to 0 °C, and then to 5 °C (not exceeding a thawing rate of 6 K h^{-1}). Leaf damage was determined visually after incubation in distilled water for 24 h at room temperature. A leaf was considered damaged when at least half of its leaf area showed a significant loss of turgor and discolouration after incubation in water; however, in most cases damage of an individual leaf was either absent or complete. Preliminary tests with *Sanguisorba minor* showed a strong correlation

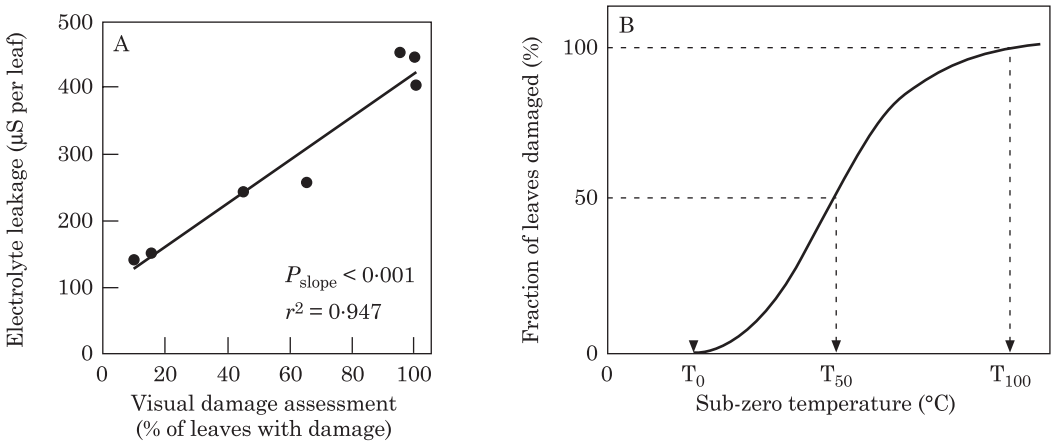

FIG. 1. A, Correlation between visual leaf damage assessment and the electrolyte leakage method for *Sanguisorba minor*. B, Three measures of freezing resistance: T_0, temperature at which initial leaf damage occurred; T_{50}, temperature at which 50 % of leaves were damaged; T_{100}, temperature at which all leaves were damaged.

between the visual assessment method and the more laborious electrolyte leakage method (Fig. 1A). For each set of ten leaves per pack, the fraction of damaged leaves was quantified and expressed as a percentage. Each of these damage percentages was plotted against its corresponding target temperature for each species and experimental plot (i.e. five points per species and experimental plot) and sigmoid curves were then visually fitted through these data points (Fig. 1B). Three different measures of freezing resistance were obtained with these sigmoid curves: (1) the lowest temperature at which no leaf in a pack was damaged (T_0); (2) the temperature at which 50 % of the leaves in a pack were damaged (T_{50}); and (3) the temperature at which all leaves in a pack were damaged (T_{100}).

Analysis of leaf chemistry

TNC (starch and soluble sugars) concentrations were determined by an enzymatic digestion procedure similar to that described by Wong (1990). Powdered plant material was boiled in distilled water for 30 min. Aliquots of the solution containing water-soluble sugars were treated with invertase and analysed for glucose using a hexosekinase reaction kit (Sigma Diagnostics, St. Louis, MO, USA). The decanted material (containing starch) was incubated with clarase (a fungal alpha amylase of *Aspergillus oryzae*, Miles Laboratory Inc., Elkhart, IN, USA) for 15 h at 40 °C and then analysed for glucose as described above. Standards of starch and sugars, as well as plant powder standards, were used as controls throughout the analysis (for further details see Körner and Miglietta, 1994; Körner *et al.*, 1995).

Statistical analysis

The effects of CO_2 (two levels) and species (five levels) on each of the three measures of leaf freezing resistance (T_0, T_{50} and T_{100}) and leaf carbohydrate parameters (soluble sugar, starch and overall TNC concentrations) were analysed by Type III ANOVA (Stata version 6, Stata Corporation, College Station, TX, USA) with $n = 4$. To assess the relationships between measures of leaf freezing resistance and leaf sugar, starch and overall TNC concentrations, linear regression models for each measure of freezing resistance were fitted through all data points (ambient and elevated CO_2 and all species). All error estimates in the text and error bars in figures are s.e.m. Statistically significant differences are defined as $P \leqslant 0{\cdot}05$.

RESULTS AND DISCUSSION

We focused our study on late-spring (May) freezing resistance because of the frequent occurrence of subzero night-time and early morning temperatures (as low as −5 °C based on 30 years of temperature data recorded at a nearby site; Brändli, 1994) at a time of year when leaves of the dominant species are highly susceptible to freezing damage. Viewed across all five species, elevated CO_2 significantly ($P = 0{\cdot}016$) increased the mean temperature at which all leaves were damaged (T_{100}) from −9·6 °C at ambient CO_2 to −8·5 °C at elevated CO_2 (Table 1). A similar, though only marginally significant ($P = 0{\cdot}079$) reduction in mean leaf freezing resistance was observed for the temperature at which 50 % of leaves were damaged (T_{50}), from −6·7 °C at ambient CO_2 to −6·0 °C at elevated CO_2. However, T_0, the temperature at which initial leaf damage occurred, remained unaffected by elevated CO_2 ($P = 0{\cdot}328$). Thus, the hypothesized enhancement of freezing resistance under elevated CO_2 was not supported. Instead we observed a decline in freezing resistance at temperatures where substantial (T_{50}) or complete (T_{100}) leaf damage occurred. These patterns indicate that decreases in leaf freezing resistance observed under elevated CO_2 become more pronounced the stronger the freezing temperatures become ($P_{T0} = 0{\cdot}328$, $P_{T50} = 0{\cdot}079$, $P_{T100} = 0{\cdot}016$). In other words, the more severe a frost event is, the more important CO_2 effects on freezing resistance may be.

Regardless of how it was assessed (T_0, T_{50} and T_{100}), freezing resistance varied greatly among the five species tested at both CO_2 levels ($P < 0{\cdot}001$, Table 1). For

TABLE 1. *Leaf freezing resistance (T_0, T_{50} and T_{100}) and concentrations of soluble sugars, starch and overall TNC of five dominant plant species growing in communities maintained at ambient and elevated CO_2 in the sixth year of CO_2 treatment*

	T_0		T_{50}		T_{100}		TNC (% dry mass)		Starch (% dry mass)		Sugars (% dry mass)	
CO_2	Amb.	Elev.	Amb.	Elev.	Amb.	Elev.	Amb.	Elev.	Amb.	Elev.	Amb.	Elev.
Bromus	−3·0 ± 0·0	−3·5 ± 0·5	−7·1 ± 0·3	−6·7 ± 0·4	−10·7 ± 0·2	−10·1 ± 0·5	4·4 ± 0·3	6·2 ± 0·6	2·8 ± 0·1	4·1 ± 0·2	1·5 ± 0·3	2·1 ± 0·3
Cirsium	−3·3 ± 0·9	−3·2 ± 0·7	−5·7 ± 0·4	−4·8 ± 0·7	−7·7 ± 0·7	−6·8 ± 0·9	3·5 ± 1·0	5·3 ± 0·8	2·0 ± 0·5	2·3 ± 0·3	1·4 ± 0·5	2·9 ± 1·0
Salvia	−2·9 ± 0·5	−1·5 ± 0·5	−4·7 ± 0·2	−3·3 ± 0·8	−7·5 ± 0·5	−5·3 ± 1·2	6·5 ± 1·2	8·2 ± 0·7	3·3 ± 0·8	4·4 ± 0·5	3·1 ± 0·5	3·9 ± 0·2
Sanguisorba	−7·6 ± 0·8	−7·3 ± 0·8	−9·8 ± 0·6	−9·6 ± 0·7	−12·4 ± 0·4	−12·7 ± 0·4	9·9 ± 0·2	14·2 ± 1·5	3·2 ± 0·4	6·9 ± 1·0	6·8 ± 0·2	7·4 ± 0·5
Trifolium	−2·0 ± 1·5	−4·0 ± 1·0	−6·3 ± 0·8	−5·7 ± 0·9	−9·7 ± 0·7	−7·5 ± 1·0	5·6 ± 0·5	8·0 ± 0·7	4·5 ± 0·5	7·0 ± 0·6	1·1 ± 0·1	1·0 ± 0·3
Average (5 species)	−3·8 ± 1·0	−3·9 ± 1·0	−6·7 ± 0·9	−6·0 ± 1·1	−9·6 ± 0·9	−8·5 ± 1·3	6·0 ± 1·1	8·4 ± 1·6	3·2 ± 0·4	4·9 ± 0·9	2·8 ± 1·1	3·5 ± 1·1
Relative change under elevated CO_2							+40 %		+53 %		+25 %	
Results of ANOVAs:												
Factor	d.f.	P		P		P	P		P		P	
CO_2	1	0·822		0·079		0·016*	< 0·001***		< 0·001***		0·042*	
Species	4	< 0·001***		< 0·001***		< 0·001***	< 0·001***		< 0·001***		< 0·001***	
CO_2 × species	4	0·328		0·879		0·309	0·523		0·053		0·710	

*$P < 0·05$; **$P < 0·01$; ***$P < 0·001$.

T_0, Temperature at which initial leaf damage occurred; T_{50}, temperature at which 50 % of leaves were damaged; T_{100}, temperature at which all leaves were damaged. Data are the means ± s.e. with $n = 4$.

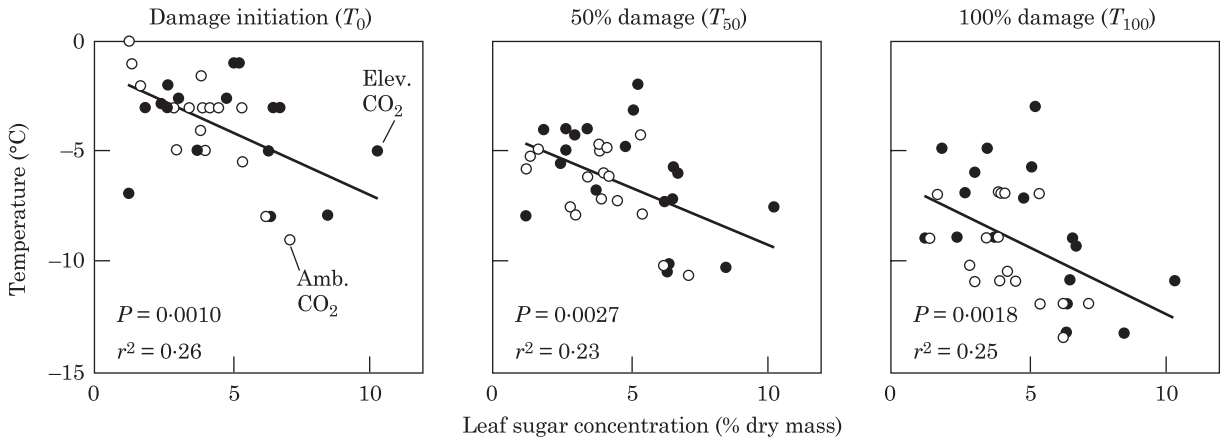

FIG. 2. Correlations between the three measures of leaf freezing resistance (T_0, T_{50} and T_{100}) and leaf sugar concentration across all five species tested for both ambient (○) and elevated (●) CO_2 (four points per species and CO_2 treatment).

example, in *Trifolium medium*, initial damage (T_0) occurred at −2 °C, whereas *Sanguisorba minor* tolerated temperatures as low as −7 °C before leaves showed any damage. Similarly, the temperature at which all leaves were damaged (T_{100}) in *Sanguisorba minor* (< −12 °C) was more than 4 K colder than the temperature needed to cause 100 % damage of *Cirsium acaule* and *Salvia pratensis* leaves. Although no significant species × CO_2 interactions were found for any measure of freezing resistance, a decrease in freezing resistance under elevated CO_2 may still have different consequences for different species. For example, a CO_2-induced decrease in freezing resistance would place *Salvia pratensis*, a species with inherently weak freezing resistance, at greater risk than a species with inherently higher freezing resistance (e.g. *Sanguisorba minor*).

Our data indicating a decrease in freezing resistance under elevated CO_2 agree with the results from Lutze *et al.*'s (1998) study that reported greater damage of *Eucalyptus* leaves under elevated CO_2 after a spring frost event. However, our data contrast with results of two studies conducted with pines that report increases [Repo *et al.* (1996) in the first year of exposure to elevated CO_2] or no change [Wiemken *et al.* (1996), in the second year of exposure; Repo *et al.* (1996) in the second and third year of exposure] in freezing resistance. The contrasting responses of freezing resistance among these studies may be due to many factors. These include the use of different plant species (e.g. trees *vs.* herbaceous species; conifers *vs.* deciduous trees); use of container-grown *vs. in situ* plant material; plant age and phenological stage; length of exposure to elevated CO_2; and how freezing resistance (or damage) was defined and reported. Repo *et al.* (1996) also observed that CO_2-induced changes in plant phenology (i.e. timing of bud-break and tissue frost hardening) contributed to the CO_2 effect on freezing resistance. However, data from our grassland indicate that elevated CO_2 had no effect on plant phenology (M. Berger and B. Thürig, unpubl. res.), suggesting that the changes in freezing resistance we observed might be a direct effect of changes in leaf chemistry.

As expected, concentrations of leaf soluble sugars (+25 %, $P = 0{\cdot}042$), starch (+53 %, $P < 0{\cdot}001$), and overall TNC (+40 %, $P < 0{\cdot}001$) were significantly higher under elevated than under ambient CO_2 across all species examined (Table 1). These results from the sixth year of CO_2 enrichment indicate that CO_2-induced increases in leaf carbohydrates persist for many years in this plant community, as has been observed in other native systems (Körner and Miglietta, 1994; Schäppi and Körner, 1997). Although carbohydrates other than starch and soluble sugars were not analysed in the sixth year, data from the previous year showed no changes in the concentrations of fructans or minor sugar fractions (e.g. sorbitol, myo inositol and pinitol) under elevated CO_2 (data not shown).

Viewed across all five species from the plant community, the temperatures at which initial (T_0), 50 % (T_{50}) and 100 % (T_{100}) leaf damage occurred decreased with increasing leaf sugar concentrations ($P \leqslant 0{\cdot}0027$, Fig. 2). In other words, leaf freezing resistance increased with increasing sugar concentrations. However, leaf sugar concentrations explained only a small part of the overall variation in T_0, T_{50} and T_{100} (r^2 values from 0·23 to 0·26), and the relationships were mainly due to one species (*Sanguisorba minor*) which had especially high leaf sugar concentrations and a correspondingly high freezing resistance (i.e. lower damage temperatures for T_0, T_{50} and T_{100}, Table 1). Neither leaf starch nor overall TNC concentrations were related to T_0, T_{50} or T_{100}.

Despite the overall relationships seen between leaf sugar concentrations and freezing resistance across all five species (Fig. 2), we found no such relationships within individual species. Furthermore, CO_2-induced enhancement of leaf sugar concentrations did not result in higher freezing resistance in any of the five species. These data suggest that the changes in sugar concentrations we observed under elevated CO_2 were too small to affect leaf freezing resistance, or that CO_2 enrichment may reduce freezing resistance in other ways that negate any potentially beneficial effect of increased sugar concentrations. One such mechanism may be a reduction in nitrogen-containing, membrane stabilizing or osmotically active antifreeze

compounds (cf. Sakai and Larcher, 1987) in leaves produced under elevated CO_2 resulting from reductions in leaf nitrogen levels [as is often observed (Sage *et al.*, 1989; Stitt, 1991), and was also seen at our study site (data not shown)].

Thus the results of our study indicate that rising atmospheric CO_2 is unlikely to enhance leaf freezing resistance in the dominant species in this native grassland. Instead, elevated CO_2 may actually increase the risk of late spring freezing damage. Although concentrations of soluble sugars, starch and overall TNC increased under elevated CO_2 in all species tested, we found no evidence that these changes were related to the shifts in freezing resistance we observed. The strong differences in freezing resistance observed among the species, along with the general decrease in leaf freezing resistance, may increase the risk of losing species that have inherently weak freezing resistances from the plant community.

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