RESEARCH ARTICLE

American Journal of Botany

BRIEF COMMUNICATION

Freeze-induced cyanide toxicity does not maintain the cyanogenesis polymorphism in white clover (*Trifolium repens*)

Nicholas J. Kooyers[1,2], Bradley Hartman Bakken[3], Mark C. Ungerer[3], and Kenneth M. Olsen[4,5]

Manuscript received 24 January 2018; revision accepted 26 April 2018.

[1] Department of Biology, University of Louisiana, Lafayette, LA 70504, USA

[2] Department of Integrative Biology, University of South Florida, Tampa, FL 33620, USA

[3] Division of Biology, Kansas State University, Manhattan, KS 66506, USA

[4] Department of Biology, Washington University, St. Louis, MO 63130, USA

[5] Author for correspondence (e-mail: kolsen@wustl.edu)

Citation: Kooyers, N. J., B. Hartman Bakken, M. C. Ungerer, and K. M. Olsen. 2018. Freeze-induced cyanide toxicity does not maintain the cyanogenesis polymorphism in white clover (*Trifolium repens*). *American Journal of Botany* 105(7): 1224–1231.

doi:10.1002/ajb2.1134

PREMISE OF THE STUDY: The maintenance of adaptive polymorphisms within species requires fitness trade-offs reflecting selection for each morph. Cyanogenesis, the ability to produce hydrogen cyanide (HCN) after tissue damage, occurs in >3000 plant species and exists as a discrete polymorphism in white clover. This polymorphism is spatially distributed in recurrent clines, with higher frequencies of cyanogenic plants in warmer climates. The HCN autotoxicity hypothesis proposes that cyanogenic plants are selected against where frosts are common, as freezing liberates HCN and could impair cellular respiration.

METHODS: We tested the HCN autotoxicity hypothesis using a freezing chamber to examine survival, tissue damage, and physiological recovery as assessed via chlorophyll fluorescence following mild and severe freezing treatments. We utilized 65 genotypes from a single polymorphic population to eliminate effects of population structure.

KEY RESULTS: Cyanogenic plants did not differ from acyanogenic plants in survival, tissue damage, or recovery following freezing. However, plants producing either of the two required cyanogenic precursors had lower survival and tissue damage after freezing than plants lacking both precursors.

CONCLUSIONS: These results suggest that freezing-induced HCN toxicity is unlikely to be responsible for the maintenance of the cyanogenesis polymorphism in white clover. However, energetic trade-offs associated with costs of producing the cyanogenic precursors may confer a fitness benefit to acyanogenic plants under stressful climatic conditions. The lack of evidence for HCN toxicity suggests that cyanogenic clover uses physiological mechanisms mediated by β-cyanoalanine synthase and alternative oxidase to maintain cellular function in the presence of HCN.

KEY WORDS adaptive clines; alternative oxidase (AOX); β-cyanoalanine synthase (β-CAS); cyanogenic glucosides; Fabaceae; freezing tolerance; *Trifolium repens*; white clover.

Cyanogenesis, the ability to produce hydrogen cyanide (HCN) upon tissue damage, is found in over 3000 species across 130 plant families (Gleadow and Møller, 2014) and can function as an effective chemical defense against herbivores (Jones, 1988; Gleadow and Møller, 2014). While the cyanogenic response varies quantitatively in many species, it occurs as a discrete Mendelian polymorphism in a limited number of species, including white clover (*Trifolium repens*), where it is characterized by cyanogenic and acyanogenic

morphs (Armstrong et al., 1913; Ware, 1925; Daday, 1954). Such Mendelian polymorphisms have historically allowed researchers to better test hypothesized ecological trade-offs that may maintain adaptive variation within and across populations (Dobzhansky, 1948; Cain and Sheppard, 1950; Kettlewell, 1955; Reznick and Travis, 1996), including plant chemical defense polymorphisms (Linhart and Thompson, 1999; Ahern and Whitney, 2014). Indeed, the cyanogenesis polymorphism in white clover has become a textbook example of how adaptive variation is maintained in natural populations (Silvertown and Charlesworth, 2001; Briggs and Walters, 2016; Futuyma and Kirkpatrick, 2017).

Cyanogenesis in white clover occurs through the interaction of two cyanogenic precursors that are separated in intact tissue and brought together following cell rupture: cyanogenic glucosides (stored in cell vacuoles), and their hydrolyzing enzyme linamarase (present in the apoplast) (reviewed by Hughes [1991]). The polymorphism arises through two independently segregating polymorphisms for each precursor, with the presence/absence of cyanogenic glucosides and linamarase controlled by the genes *Ac/ac* and *Li/li*, respectively (Coop, 1940; Melville and Doak, 1940; Corkill, 1942). For both *Ac/ac* and *Li/li*, the dominant allele confers the presence of the cyanogenic precursor, and recessive alleles correspond to gene deletions (Olsen et al., 2007, 2008, 2013; Olsen and Small 2018). Thus, four different cyanogenesis phenotypes (or "cyanotypes") can be found in nature: plants with at least one dominant allele at both genes are cyanogenic (AcLi), whereas acyanogenic plants may lack either cyanogenic glucosides (acLi), or linamarase (Acli) or both components (acli).

While most populations across the circumglobal range of white clover have been found to have both cyanogenic and acyanogenic morphs, strong clines in the frequency of cyanogenic plants exist across gradients in latitude (Daday, 1954, 1958; Kooyers and Olsen, 2012), elevation (Daday, 1954; Ganders, 1990; Kooyers and Olsen, 2013), and urban–rural transects (Thompson et al., 2016). The existence of these recurrently evolved clines suggests that spatial heterogeneity in abiotic or biotic factors across these gradients creates strong selection for cyanogenic or acyanogenic morphs. Multiple different ecological factors have been implicated in generating these clines, including spatially varying herbivore pressure (reviews by Hughes, 1991; Kooyers et al., 2014), fungal susceptibility (Dirzo and Harper, 1982), water availability (Kooyers and Olsen, 2013; Kooyers et al., 2014), resource allocation costs (Kakes, 1989), and freezing tolerance (Daday, 1965; Foulds and Young, 1977).

In his classic studies documenting worldwide cyanogenesis clines, Daday (1954, 1958, 1965) proposed that the climate-associated clines primarily reflect the physiological costs of cyanogenesis in frost-prone environments. Specifically, he invoked a potential mechanism that has subsequently been referred to as HCN autotoxicity; since freezing ruptures cells, it could in principle release toxic levels of HCN into the tissue of cyanogenic plants. HCN inhibits cytochrome *c* oxidase in the electron transport chain, thereby shutting down cellular respiration and ATP synthesis (Antonini et al., 1971; Cooper and Brown, 2008), and it is also a potent inhibitor of photosynthesis (Foulds and Young, 1977). In support of the HCN autotoxicity hypothesis, Daday (1965) reported that acyanogenic acli plants showed less evidence of frost injury than cyanogenic (AcLi) plants when grown under natural alpine conditions. Daday considered any role of cyanogenesis as an anti-herbivore defense to be at most a secondary factor in cyanogenesis cline evolution (Daday 1958, 1965).

The field of plant physiology has advanced considerably since the HCN autotoxicity hypothesis was proposed a half century ago, and plants are now known to have metabolic processes to detoxify cellular HCN and maintain respiration in its presence (Taiz et al., 2015). The existence of these pathways calls into question the plausibility of the HCN autotoxicity hypothesis. HCN is naturally produced at low levels in all plant species as a byproduct of ethylene biosynthesis (Peiser et al., 1984; Abeles, 2012), and HCN detoxification pathways (mediated by β-cyanoalanine synthase, or β-CAS) have been described in multiple plant species including white clover (Miller and Conn, 1980; Yu, 2015; Machingura et al., 2016). Moreover, in some cyanogenic species, the enzymes involved in cyanide detoxification have been found to show elevated activity in tissues with greater cyanogenic glucoside concentrations (Mizutani et al., 1991), suggesting that cyanogenic species possess the physiological mechanisms to effectively detoxify excess cellular HCN. In addition to HCN detoxification pathways, there are also now well-described mechanisms by which cellular respiration is maintained in the presence of HCN through cyanide-resistant alternative oxidase (AOX)-mediated respiration (Henry and Nyns, 1975; Piotrowski et al., 2001; Vanlerberghe, 2013). Given these mechanisms of HCN metabolism in plants, the extent to which freezing-induced cyanogenesis would be expected to result in any tissue injury in white clover is unclear.

In the decades since Daday's early studies of cyanogenesis clines, a substantial body of evidence has been generated in support of the hypothesis that cyanogenesis functions as a chemical defense and that cyanogenic clover plants are selectively favored where generalist herbivores are abundant (reviewed by Hughes, 1991 and Olsen et al., 2013). Fewer studies have examined Daday's original hypothesis that cyanogenic plants suffer differential physiological damage in frost-prone regions. Nonetheless, this explanation continues to be invoked as a likely selective mechanism (e.g., Thompson et al., 2016). While some observational studies and manipulative experiments have found correlations between cyanogenesis and freezing-induced tissue damage (Caradus et al., 1989; Caradus and Eerens, 1992), others report no difference between morphs in cellular respiration (Foulds and Young, 1977) or tissue damage (Olsen and Ungerer, 2008) following freezing. One explanation for these equivocal results is that most studies have relied on limited sampling designs that either examine a small number of genotypes, which restricts statistical power, or sample many genotypes from across a range of climates, which potentially confounds cyanogenesis with unrelated determinants of climatic adaptation. The latter sampling design is problematic as it risks generating spurious correlations between freeze tolerance and cyanogenesis—since most acyanogenic plants come from regions with colder climates and thus are inherently more likely to be frost tolerant (discussed by Olsen and Ungerer, 2008). In addition, the experimental designs of previous freezing tolerance studies have not distinguished among acyanogenic cyanotypes (Acli, acLi, acli), which does not allow for the fitness costs of HCN production to be distinguished from potential costs of producing the two required precursors.

In this study, we attempt to overcome the sampling limitations of earlier studies and test the HCN autotoxicity hypothesis by conducting a manipulative experiment utilizing a large number of accessions from a single locality in the center of a latitudinal cyanogenesis cline. We specifically tested whether cyanogenic plants (1) exhibit greater levels of freezing-induced tissue damage than acyanogenic plants after exposure to biologically relevant freezing

temperatures and (2) show evidence of inhibited physiological recovery following exposure to freezing. Our results suggest that cyanogenic plants are not differentially susceptible to freezing damage and that other factors, including those related to the energetic costs of producing cyanogenic precursors, are more likely to maintain the cyanogenesis polymorphism and climate-associated cyanogenesis clines.

MATERIALS AND METHODS

Germplasm

The experiment utilized 65 genotypes originating from cuttings taken from field populations in Grenada, Mississippi (33°46′30″N, 89°48′32″W), where all four cyanotypes are present at moderate frequencies: 42% AcLi, 13% Acli, 27% acLi, and 18% acli (Kooyers and Olsen, 2012). Cyanotypes for the selected accessions were previously determined using Feigl-Anger phenotyping for the presence/absence of each cyanogenic component and PCR-genotyping of the underlying cyanogenesis genes (*CYP79D15* underlying *Ac/ac* and *Li* underlying *Li/li*) (Kooyers and Olsen, 2012). Among the selected set of tested genotypes, the cyanotype proportions were approximately equal: 17 AcLi, 16 Acli, 16 acLi, and 16 acli plants. Stolon cuttings were taken after plants had been growing in the greenhouse at Washington University in St. Louis for at least 6 months. Three replicate clones were established per genotype. Each clone was established from one cutting and allowed to grow under standard greenhouse conditions for 60 days before experiments began.

Freezing tolerance experiments

Freezing experiments tested two minimum temperatures that were selected based on results of preliminary trials. The milder treatment (−6°C) was selected as the temperature where greatest variation in freezing-induced tissue damage was observed among genotypes; the more severe treatment (−10°C) was selected as the temperature where aboveground biomass did not survive for ~50% of plants in the preliminary trial. These temperatures also accurately reflect climatic conditions in the source population. The average low temperature during January in Grenada, MS is −1°C, and average extreme minimum winter temperature −12.8°C. The historic low temperature for the collection location is −24.4°C (National Climatic Data Center; www.ncdc.noaa.gov).

Freezing trials were conducted in a programmable environmental chamber (ESU-3CA; Espec) in the Division of Biology at Kansas State University. The overall experimental regime was the same for both freezing treatments. Photoperiod was constant at a 12-h light:12-h dark cycle, and plants were kept well watered throughout the experiment. Plants were cold-acclimated for 7 d at 4°C in a cold room. On the night of the eighth day, plants were placed in the environmental chamber where they experienced a temperature decrease of −2°C per hour down to the minimum temperature (either −6°C or −10°C). Plants were held at the minimum temperature for 2.5 h before the temperature began to increase at a rate of 2°C/h. The same cycle was repeated on the ninth day. After the temperature rose to 4°C on the morning of the 10th day, plants were returned to the cold room for 24 h, then transferred to a growth room (21°C) for 3 d before assessment of damage. The −6°C treatment was performed first, and plants were given between 49–56 d to recover before the next cold acclimation treatment began. Some clones were excluded from the −10°C treatment (32/195); however, each genotype was still represented. Since the freezing chamber was not large enough to accommodate all plants at once, plants were randomly assigned to six different blocks that were assayed consecutively.

Freezing damage metrics and statistics

Freezing damage was assessed in three ways. First, survival was determined by visually assessing whether plants had any surviving aboveground tissue. Plants with no surviving tissue were considered dead. None of these plants recovered after they were returned to greenhouse conditions. Generalized linear mixed models implemented in the lme4 package version 1.1-10 (Bates et al., 2014) were used to analyze the survival data in R 2.13.2 (R Foundation for Statistical Computing, Vienna, Austria). Models included presence/absence of cyanogenic glucosides and linamarase and their interaction as separate fixed factors, block as a random factor and genotype as a random factor. Survival was modeled using a binomial distribution and logit link. Statistical significance of factors and interactions were tested using ANOVA with type III sum of squares and a Wald chi-square test statistic implemented in the car package version 2.1-6 (Fox et al., 2013). If the HCN autotoxicity hypothesis were correct, one would expect that cyanogenic (AcLi) plants would have lower survival than acyanogenic plants (Acli, acLi, or acli cyanotypes) after freeze treatments, leading to a significant interaction between cyanogenic glucosides and linamarase with our GLMM. Alternatively, if either cyanogenic glucosides or linamarase alone impacts survival following a freeze treatment, then there may be a fitness cost to producing a cyanogenic precursor.

Second, aboveground tissue damage was assessed visually using a semi-quantitative measure that has previously been employed in studies of plant cold tolerance (Olsen and Ungerer, 2008; Zhen and Ungerer, 2008; Zhen et al., 2011). Plants were assigned a score between 0–4, indicating the level of tissue damage following freezing. Plants with <25% damage were assigned a score of 4, those with 25–50% damage were assigned a 3, those with 50–75% damage were assigned a 2, and those with 75–100% damage were assigned a 1. Plants with no surviving aboveground tissue were assigned a score of 0. To assess differences between cyanotypes, we created independent GLMM models for tissue damage for the −6°C and −10°C experiments. Models and significance testing were identical to GLMM models for survival except tissue damage was modeled using a Gaussian distribution with an identity link. If the HCN autotoxicity hypothesis were correct, one would expect that cyanogenic (AcLi) plants would have greater tissue damage than acyanogenic plants (Acli, acLi, or acli cyanotypes) following freeze treatments. Again, this difference would result in a significant interaction between cyanogenic glucosides and linamarase terms in the GLMM.

As a third measure of tissue damage, we used chlorophyll fluorescence (specifically, F_v/F_m) as an indicator of photosynthetic efficiency and plant stress after tissue damage (Maxwell and Johnson, 2000; Zhen et al., 2011). Briefly, incoming light to a plant can be used for photosynthesis, dissipated as heat, or re-emitted as fluorescence. F_v/F_m is a measure of the amount of light being used for photosynthesis vs. that emitted as fluorescence and has an optimal value of ~0.83 for most plants including white clover (Johnson et al., 1993); lower values reflecting closures of reaction centers in photosystem II and general plant stress (Maxwell and Johnson, 2000). We analyzed every plant by taking five independent measures of

F_v/F_m (with each measure made on a single green leaflet from five leaves) at three time points in the experimental regime: before cold acclimation, before entering the freeze chamber, and 3 d after freezing. All measurements were taken in the dark with a photosynthesis yield analyzer MINI-PAM (Heinz Walz, Effeltrich, Germany). The five independent measures were averaged for each time. There was a lower sample size after the −10°C treatment because many plants did not survive or had few leaves of sufficient quality. As with visual assessments of tissue damage, linear mixed models were used to assess whether cyanotypes differed in their ability to recover photosynthetic ability. Each time point was modeled separately. Fixed and random factors were the same as in the freeze damage models except for the inclusion of F_v/F_m before cold acclimation as a covariate in modeling F_v/F_m after freezing. If the HCN autotoxicity hypothesis were correct, cyanogenic (AcLi) plants would be expected to recover more slowly than acyanogenic (Acli, acLi, or acli) plants.

RESULTS

The cyanogenic phenotype does not show differential freezing damage

To determine whether acyanogenic plants had greater freezing tolerance than cyanogenic plants, we measured survival, tissue damage, and physiological recovery following freeze treatments with minimum temperatures of −6°C and −10°C. Raw data are available in the Supplemental Data with this article (Appendix S1).

Survival—All plants survived the −6°C treatment, but only 40% of all clonal replicates survived the −10°C treatment, and only one of the surviving clones had less than 75% tissue damage at this temperature. Counter to predictions of the HCN autotoxicity hypothesis, cyanogenic genotypes did not show significantly lower survival than acyanogenic genotypes in the −10°C treatment (*Ac/ac*:*Li/li*, χ^2 = 0.84, p = 0.36; Table 1, Fig. 1). While survival averaged between genotypes within a cyanotype was slightly lower for cyanogenic plants than for acyanogenic plants as a whole, the lowest survival of all was for the acyanogenic Acli cyanotype (~27%; Fig. 1; Appendix S1, see Supplemental Data), suggesting that production of hydrogen cyanide did not drive differences in survival. However, plants producing cyanogenic glucosides (AcLi and Acli phenotypes collectively) had lower survival versus plants not producing cyanogenic glucosides (acLi and acli) (*Ac/ac*; χ^2 = 4.6, p = 0.03; Fig. 1, Appendix S1).

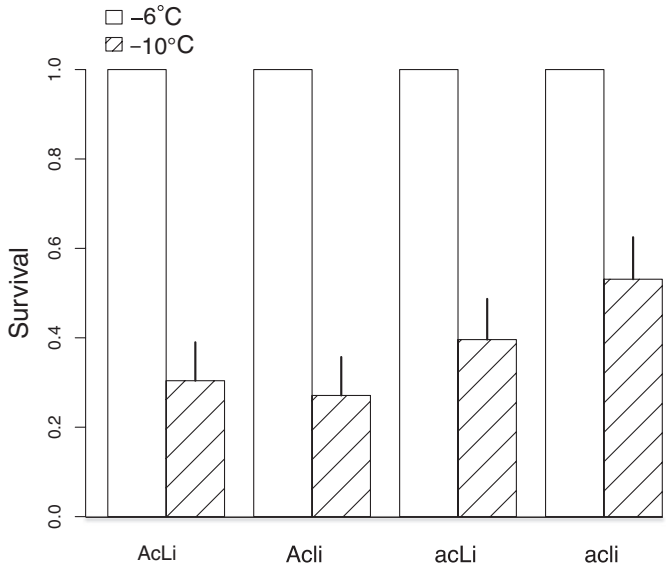

FIGURE 1. Survival following freezing treatments at either −6°C (white bars) or −10°C (striped bars) did not differ between cyanogenic and acyanogenic cyanotypes. Survival was calculated as the probability of survival averaged across all genotypes exhibiting a specific cyanotype. Error bars represent standard error around the mean.

Tissue damage—Cyanogenic and acyanogenic plants differed little in the levels of tissue damage after the −6°C treatment (*Ac/ac*:*Li/li*, χ^2 = 1.12, p = 0.36; Table 1, Fig. 2) or the −10°C treatment (*Ac/ac*:*Li/li*, χ^2 = 0.91, p = 0.34). After the −6°C treatment, cyanogenic plants (AcLi) had a mean tissue damage score of 3.04 ± 0.76, and acyanogenic plants (Acli, acLi, and acli combined) had nearly identical mean scores (3.04 ± 0.67). While there was much greater tissue damage after the −10°C treatment, the average tissue damage score for cyanogenic plants and acyanogenic plants was still very similar (AcLi: 0.36 ± 0.42; Acli, acLi, acli: 0.43 ± 0.38). As with the survival metric results, Acli plants had the most tissue damage, and producing cyanogenic glucoside resulted in significantly more tissue damage in both treatments (−6°C: *Ac/ac*, χ^2 = 3.93, p = 0.047; −10°C: *Ac/ac*, χ^2 = 4.28, p = 0.039). Although tissue damage estimates included individuals that did not survive (i.e., had no surviving tissue in the −10°C treatment), excluding these individuals from the analysis led to similar qualitative conclusions.

TABLE 1. Generalized linear mixed modeling results for morphological and physiological measures following freeze experiments.

		Model coefficients			*Ac/ac*		*Li/li*		*Ac/ac*:*Li/li*	
Response variable	**Int.**	**Ac**	**Li**	***Ac/ac*:*Li/li***	χ^2	p	χ^2	p	χ^2	p
Survival (−10°C)	0.389	−1.812	−0.610	1.003	**4.44**	**0.035**	0.63	0.427	0.84	0.361
Tissue damage (−6°C)	3.297	−0.446	−0.218	0.331	**3.93**	**0.047**	0.96	0.328	1.12	0.290
Tissue damage (−10°C)	0.566	−0.287	−0.107	0.181	**4.28**	**0.039**	0.61	0.435	0.91	0.340
F_v/F_m (−6°C, control)	0.838	−0.006	−0.004	0.010	2.09	0.148	0.82	0.366	2.82	0.093
F_v/F_m (−6°C, acclimated)	0.814	−0.001	−0.002	0.005	0.03	0.862	0.17	0.682	0.53	0.468
F_v/F_m (−6°C, recovery)	0.597	−0.005	−0.002	0.002	1.47	0.225	0.24	0.627	0.11	0.741
F_v/F_m (−10°C, control)	0.830	0.000	0.004	0.003	0.00	0.947	0.77	0.379	0.22	0.642
F_v/F_m (−10°C, acclimated)	0.813	−0.006	−0.003	0.008	1.25	0.264	0.28	0.598	1.09	0.296
F_v/F_m (−10°C, recovery)	0.506	−0.014	0.024	−0.019	0.39	0.534	1.48	0.223	0.36	0.549

Notes: Generalized linear mixed models included presence or absence of cyanogenic glucosides and linamarase and the interaction as fixed factors. Genotype and block were included as random factors. Significance of fixed factors and interaction were tested via ANOVA using a Wald χ^2 test statistic with 1 degree of freedom.

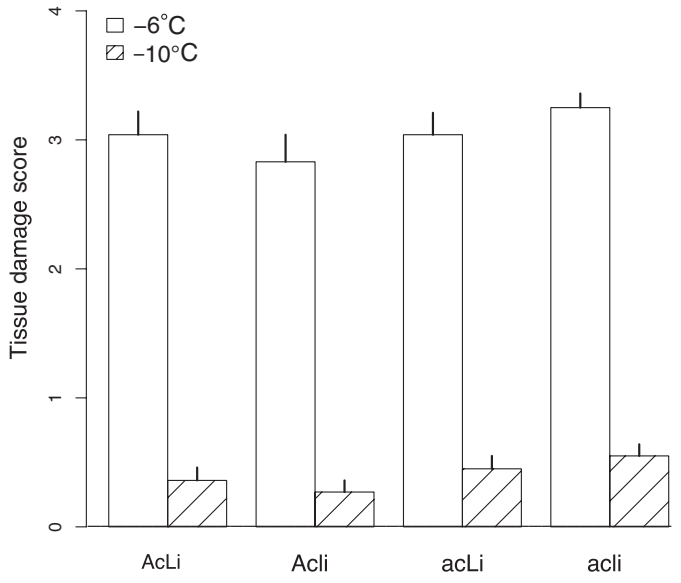

FIGURE 2. Levels of tissue damage following freezing at either −6°C (white bars) or −10°C (striped bars) do not differ between cyanogenic and acyanogenic cyanotypes. Error bars represent standard error around the mean.

Photosynthetic rate—There was little difference between cyanotypes in chlorophyll fluorescence-based measures of tissue stress either before or after the freeze treatments (Tables 1, 2). Before freezing, cold acclimation reduced the number of potential photosynthetic reaction centers available from previous greenhouse conditions, but not to a level that is typically associated with plant stress (Maxwell and Johnson, 2000).

After freezing, cyanotypes all recovered well from the −6°C treatment, with F_v/F_m measures similar to before-treatment values after 3 d of recovery; cyanogenic and acyanogenic plants did not differ in this recovery (*Ac/ac*:*Li/li*, $\chi^2 = 0.11$, $p = 0.74$). The −10°C treatment stressed all plants to a point where they were still exhibiting signatures of stress after the 3-d recovery period. However, there were no significant differences between cyanotypes in F_v/F_m (*Ac/ac*:*Li/li*, $\chi^2 = 0.36$, $p = 0.55$), and Acli plants rather than AcLi plants recovered most poorly (Table 2).

DISCUSSION

Despite more than six decades of studies examining the environmental factors that maintain the white clover cyanogenesis polymorphism, the major selective forces at play have remained unresolved. Here we have tested the earliest proposed hypothesis, that freezing temperatures in colder climates select against cyanogenic plants due to HCN autotoxicity (Daday, 1958, 1965). Our results indicate that cyanogenic white clover plants do not exhibit greater mortality, tissue damage, or physiological stress following freezing damage than acyanogenic plants. These findings indicate that freezing-induced HCN toxicity is unlikely to be a key selective factor in the evolution of cyanogenesis clines or the maintenance of the cyanogenesis polymorphism.

While freezing tolerance is not correlated with cyanogenesis, our results suggest that plants producing either cyanogenic glucosides or linamarase have lower survival following the severe freeze treatment than plants lacking both cyanogenic precursors (acli cyanotypes). Notably, this finding is entirely consistent with Daday's (1965) original experimental results, which specifically compared AcLi and acli genotypes in an alpine environment. With the benefit of comparisons among all four cyanotypes, it becomes apparent that the reduced freezing tolerance of AcLi plants is not attributable to HCN production per se, as the Acli and acLi cyanotypes—both of which are acyanogenic—also show decreased freezing tolerance (Figs. 1, 2; Table 2). Rather, our results suggest that energetic costs associated with the production of the individual cyanogenic precursors may exact a fitness cost in survival or recovery from freezing stress. Below we discuss these results in the context of prior experiments on HCN autotoxicity and cyanogenesis, the potential physiological mechanisms preventing or mitigating intracellular HCN toxicity, and the potential agents of selection that may maintain cyanogenesis clines.

While most evolutionary ecology research since Daday's studies has focused on maintenance of the cyanogenesis polymorphism through herbivore deterrence (a selective factor which Daday himself considered to be of secondary importance at best), a limited number of studies have examined freezing tolerance as related to cyanogenesis in white clover. Dirzo and Harper (1982) used experimental trials in a field setting to assess freezing tolerance as related to cyanogenesis variation. As with Daday's (1965) original freezing tolerance experiment, their field study examined only AcLi and acli cyanotypes and so would not distinguish between HCN toxicity and fitness costs of producing cyanogenic precursors. In that experiment, it is also difficult to disentangle selection due to fungal pathogens and freeze damage. Both pressures selected for acyanogenic plants during the spring and early winter, but later in the winter there was greater freeze damage on *acyanogenic* plants. These simultaneously acting agents of selection make it difficult to determine the strength and direction of selection of any single agent (Kooyers et al., 2014).

In addition to field trials, some studies have used artificial freezing experiments to examine freezing tolerance as related to cyanogenesis (Brighton and Horne, 1977; Foulds and Young, 1977;

TABLE 2. F_v/F_m summary statistics for each cyanotype spanning freezing experiments.

	−6°C			−10°C		
Cyanotype	Control Mean (SD)	Acclimated Mean (SD)	Recovery Mean (SD)	Control Mean (SD)	Acclimated Mean (SD)	Recovery Mean (SD)
AcLi	0.84 (0.01)	0.82 (0.02)	0.84 (0.02)	0.84 (0.02)	0.81 (0.02)	0.77 (0.06)
Acli	0.83 (0.02)	0.81 (0.02)	0.84 (0.01)	0.83 (0.01)	0.81 (0.01)	0.76 (0.04)
acLi	0.83 (0.01)	0.81 (0.01)	0.84 (0.01)	0.83 (0.01)	0.81 (0.01)	0.80 (0.02)
acli	0.84 (0.01)	0.81 (0.01)	0.84 (0.01)	0.83 (0.01)	0.81 (0.02)	0.76 (0.06)

Notes: F_v/F_m values reflect the average of ~15 genotypes per cyanotype. The control category refers to F_v/F_m measurement before cold acclimation; the acclimated category refers to measurements after plants have been cold acclimated for 8 d at 4°C. Recovery measurements were taken 4 days after each freeze treatment.

Caradus et al., 1989; Caradus and Eerens, 1992; Olsen and Ungerer, 2008). Brighton and Horne (1977) found impaired cellular respiration in cyanogenic morphs relative to acyanogenic morphs of *Lotus corniculatus* (another polymorphic legume species) after freezing. In contrast, Foulds and Young (1977) did not find such effects in either *L. corniculatus* or white clover in a comparable freezing experiment. Caradus and colleagues (Caradus et al., 1989; Caradus and Eerens, 1992) detected weak but significant correlations between freezing tolerance and cyanogenesis in freezing tolerance trials using collections of white clover breeding lines and cultivars. However, their cold hardiness experiments were not designed to test for a causal relationship between cyanogenesis and freezing tolerance, and because acyanogenic genotypes tend to occur predominantly in colder climates, the effects of cold adaptation and cyanogenesis would have been confounded in the tested lines. To control for such climate-of-origin effects, Olsen and Ungerer (2008) compared freezing tolerance between cyanogenic and acyanogenic plants sampled from three polymorphic populations; no significant effects of cyanotype on freezing tolerance were detected once geographical origin was taken into account. As with the field experiments described above, none of these controlled freezing experiments examined variation among acyanogenic cyanotypes (Acli, acLi, acli); thus, any fitness effects of HCN toxicity would have been indistinguishable from the fitness costs of producing the cyanogenic precursors.

Rejection of the HCN autotoxicity hypothesis makes sense in light of the current understanding of plant physiology and HCN metabolism. Most research on HCN toxicity in white clover came before the knowledge that all plants produce HCN in small amounts as a byproduct of ethylene biosynthesis (Peiser et al., 1984; Abeles, 2012) and that all surveyed plant species possess the ability to conduct cellular respiration in the presence of HCN as well as to detoxify HCN. Hydrogen cyanide is toxic to many organisms because it inhibits cytochrome *c* oxidase, blocking the electron transport chain and preventing cellular respiration. Plants, including white clover, possess the mitochondrial enzyme alternative oxidase (AOX) that provides a similar function to cytochrome *c* oxidase (catalyzing the oxidation of quinol and the reduction of oxygen to water) (Henry and Nyns, 1975; Vanlerberghe et al., 1997; McDonald, 2008). AOX is sufficient to support respiratory carbon metabolism when the cytochrome pathway is inhibited by cyanide (Vanlerberghe et al., 1997) and is thought to maintain homeostasis during biotic and abiotic stress (Vanlerberghe, 2013). However, unlike cytochrome *c* oxidase, AOX does not pump protons or lead to the creation of ATP.

Plants also possess mechanisms of HCN detoxification so that HCN does not accumulate in deleterious amounts (Blumenthal et al., 1963; Yu, 2015; Machingura et al., 2016). Most HCN in cyanogenic plants is detoxified through the β-cyanoalanine synthesis pathway (Gleadow and Møller, 2014; Machingura et al., 2016). In this pathway, cyanoalanine synthase converts HCN and cysteine into the less-reactive cyanoalanine (Blumenthal et al., 1968). Cyanoalanine can then be converted into asparagine by cyanoalanine hydratase (Piotrowski et al., 2001; Piotrowski and Volmer, 2006). Although the amount of HCN released in highly cyanogenic plants following tissue damage (up to 350 mg/g in white clover; Poulton, 1990) is likely orders of magnitude higher than the HCN released during maximum ethylene biosynthesis, it is likely detoxified via the same pathway. In multiple cyanogenic species, including white clover, activities of the enzymes involved in the β-cyanoalanine synthesis pathway have been shown to have higher activity either in tissues with higher levels of cyanogenic glucosides or in species with higher HCN potential, suggesting that this pathway is active in the continuous turnover of HCN (Miller and Conn, 1980; Mizutani et al., 1991). Thus, due to the existence of AOX-based mechanisms of tolerance of HCN and β-cyanoalanine-synthesis-based detoxification methods for HCN, the autotoxicity hypothesis seems increasingly implausible. Indeed, to our knowledge, no study has ever documented physiological damage in a plant due to endogenous HCN liberation.

While our study does not support the HCN autotoxicity hypothesis, the variation observed in freezing tolerance among cyanotypes (most notably in survival and tissue damage after the −10° C treatment; Figs. 1, 2) is entirely consistent with climate-associated fitness trade-offs related to the energetic costs of producing the cyanogenic precursors. Plants producing cyanogenic glucosides (AcLi and Acli cyanotypes) were significantly less likely to survive severe freezing than cyanotypes not producing cyanogenic glucosides (Fig. 1; Appendix S1) and also had more tissue damage after both mild and severe freezing (Fig. 2; Appendix S1). Likewise, plants producing linamarase only (acLi cyanotypes) showed 13% higher mortality at the −10°C treatment than plants producing neither precursor (acli cyanotypes). These patterns suggest that producing and/or storing either cyanogenic precursor can incur a cost for the plant in stressful environments. It is interesting that plants that produce both cyanogenic glucosides and linamarase (AcLi plants) do not have lower survival or more tissue damage than plants producing only one of these compounds (Acli or acLi) as would be expected if both compounds have energetic costs. One potential explanation is that we could not detect whether each AcLi, Acli, or acLi genotype was homozygous or heterozygous for the *Ac* or *Li* alleles, a distinction that changes the quantitative level of cyanogenic glucosides or linamarase, and could modify our expectations for the energetic costs of each cyanotype.

While fitness trade-offs associated with the production of these compounds has not previously been documented in the context of freezing tolerance, previous research has documented evidence of energetic costs associated with their production in other contexts. Numerous studies have demonstrated that cyanogenic plants are differentially protected from grazing generalist herbivores (reviewed by Hughes, 1991 and Olsen et al., 2013). At the same time, plants lacking one or both cyanogenic precursors have been found to show greater vegetative growth and reproductive output in common garden experiments (Kakes, 1989; but see Thompson and Johnson, 2016; Wright et al., 2017). Similar resource allocation costs to producing cyanogenic glucosides and linamarase have been also been documented in growth chamber experiments under various combinations of low fertilizer and water availability (Kooyers et al., 2014). Collectively, these results indicate that energetic costs of producing the components required for cyanogenesis may be higher under conditions of abiotic stress, but that the protective benefits of cyanogenesis outweigh these costs in areas of high herbivore pressure.

In the context of cyanogenesis cline evolution, our results suggest that geographical variation in freezing stress may be an important selective factor in generating the climate-associated cyanogenesis clines that have evolved in white clover populations worldwide. Freezing stress is particularly interesting in the formation of parallel cyanogenesis clines across urban–rural gradients, i.e., higher frequency of cyanogenic plants in rural than urban areas, because other proposed agents of selection do not appear to vary across these gradients (Thompson et al., 2016). We suggest that

cyanogenesis clines may be driven by a resource allocation tradeoff where environments with colder temperatures select for plants that allocate fewer resources to cyanogenesis. However, there is currently not sufficient evidence to indicate that any given proposed agent of selection is adequate to create or maintain cyanogenesis clines. Future studies must work to document the full range of environmental gradients that vary across clines and design manipulative experiments identify the selective mechanisms and ecological interactions underlying cline formation.

ACKNOWLEDGEMENTS

The authors thank the Washington University in St. Louis greenhouse staff for plant growth assistance. Helpful comments on this manuscript were provided by two anonymous reviewers. Support for this work was provided by Washington University in St. Louis, a NSF Doctoral Dissertation Improvement Grant to N.J.K. (DEB-1110588) and NSF grants (DEB-0845497 & IOS-1557770) to K.M.O.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

LITERATURE CITED

Abeles, F. B. 2012. Ethylene in plant biology. Elsevier, St. Louis, MO, USA.

Ahern, J. R., and K. D. Whitney. 2014. Sesquiterpene lactone stereochemistry influences herbivore resistance and plant fitness in the field. *Annals of Botany* 113: 731–740.

Antonini, E., M. Brunori, G. C. Rotilio, C. Greenwood, and B. G. Malmström. 1971. The interaction of cyanide with cytochrome oxidase. *FEBS Journal* 23: 396–400.

Armstrong, H. E., E. F. Armstrong, and E. Horton. 1913. Herbage Studies. II.-Variation in *Lotus corniculatus* and *Trifolium repens*: cyanophoric plants. *Proceedings of the Royal Society, B, Biological Sciences* 86: 262–269.

Bates, D., M. Machler, B. M. Bolker, and S. Walker. 2014. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67: 1–48.

Blumenthal, S., G. Butler, and E. Conn. 1963. Incorporation of hydrocyanic acid labelled with carbon-14 into asparagine in seedlings. *Nature* 197: 718–719.

Blumenthal, S., H. Hendrickson, Y. Abrol, and E. Conn. 1968. Cyanide metabolism in higher plants. 3. The biosynthesis of β-cyanolanine. *Journal of Biological Chemistry* 243: 5302–5307.

Briggs, D., and M. B. Walters. 2016. Plant variation and evolution, 4th ed. Cambridge University Press, Cambridge, UK.

Brighton, F., and M. T. Horne. 1977. Influence of temperature on cyanogenic polymorphisms. *Nature* 265: 437–438.

Cain, A. J., and P. M. Sheppard. 1950. Selection in the polymorphic land snail *Cepaea nemoralis*. *Heredity* 4: 275–294.

Caradus, J., A. C. Mackay, J. Van Den Bosch, D. Greer, and G. S. Wewala. 1989. Intraspecific variation for frost hardiness in white clover. *Journal of Agricultural Science* 112: 151–157.

Caradus, J. R., and J. P. J. Eerens. 1992. Genetic adaptation to frost tolerance in white clover. *Agronomy NZ Journal* 22: 103–109.

Coop, I. 1940. Cyanogenesis in white clover III. Study of linamarase. *New Zealand Journal of Science and Technology* 22: 71–83.

Cooper, C. E., and G. C. Brown. 2008. The inhibition of mitochondrial cytochrome oxidase by the gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide: chemical mechanism and physiological significance. *Journal of Bioenergetics and Biomembranes* 40: 533–539.

Corkill, L. 1942. Cyanogenesis in white clover (*Trifolium repens* L.) V. The inheritance of cyanogenesis. *New Zealand Journal of Science and Technology* 23: 178–193.

Daday, H. 1954. Gene frequencies in wild populations of *Trifolium repens* I. Distribution by latitude. *Heredity* 8: 377–384.

Daday, H. 1958. Gene frequencies in wild populations of *Trifolium repens* L. III. World distribution. *Heredity* 12: 169–184.

Daday, H. 1965. Gene frequencies in wild populations of *Trifolium repens* L. IV. Mechanism of natural selection. *Heredity* 20: 355–365.

Dirzo, R., and J. L. Harper. 1982. Experimental studies on slug–plant interactions: IV. The performance of cyanogenic and acyanogenic morphs of *Trifolium repens* in the field. *Journal of Ecology* 70: 119–138.

Dobzhansky, T. 1948. Genetics of natural populations. XVI. Altitudinal and seasonal changes produced by natural selection in certain populations of *Drosophila pseudoobscura* and *Drosophila persimilis*. *Genetics* 33: 158.

Foulds, W., and L. Young. 1977. Effect of frosting, moisture stress and potassium cyanide on the metabolism of cyanogenic and acyanogenic phenotypes of *Lotus corniculatus* L. and *Trifolium repens* L. *Heredity* 38: 19–24.

Fox, J., M. Friendly, and S. Weisberg. 2013. Hypothesis tests for multivariate linear models using the car package. *R Journal* 5: 39–52.

Futuyma, D. J., and M. Kirkpatrick. 2017. Evolution, 4th ed. Sinauer, NY, NY, USA.

Ganders, F. R. 1990. Altitudinal clines for cyanogenesis in introduced populations of white clover near Vancouver, Canada. *Heredity* 64: 387–390.

Gleadow, R. M., and B. L. Møller. 2014. Cyanogenic glycosides: synthesis, physiology, and phenotypic plasticity. *Annual Review of Plant Biology* 65: 155–185.

Henry, M., and E. Nyns. 1975. Cyanide-insensitive respiration. An alternative mitochondrial pathway. *Subcellular Biochemistry* 4: 1–65.

Hughes, M. A. 1991. The cyanogenic polymorphism in *Trifolium repens* L. (white clover). *Heredity* 66: 105–115.

Johnson, G. N., A. J. Young, J. D. Scholes, and P. Horton. 1993. The dissipation of excess excitation energy in British plant species. *Plant, Cell & Environment* 16: 673–679.

Jones, D. 1988. Cyanogenesis in animal–plant interactions. *Ciba Foundation Symposium* 140: 151–170.

Kakes, P. 1989. An analysis of the costs and benefits of the cyanogenic system in *Trifolium repens* L. *Theoretical and Applied Genetics* 77: 111–118.

Kettlewell, H. B. D. 1955. Selection experiments on the industrial melanism in the *Lepidotera*. *Heredity* 9: 323–342.

Kooyers, N. J., L. R. Gage, A. Al-Lozi, and K. M. Olsen. 2014. Aridity shapes cyanogenesis cline evolution in white clover (*Trifolium repens* L.). *Molecular Ecology* 23: 1053–1070.

Kooyers, N. J., and K. M. Olsen. 2012. Rapid evolution of an adaptive cyanogenesis cline in introduced North American white clover (*Trifolium repens* L.). *Molecular Ecology* 21: 2455–2468.

Kooyers, N. J., and K. M. Olsen. 2013. Searching for the bull's eye: Agents and targets of selection vary among geographically disparate cyanogenesis clines in white clover (*Trifolium repens* L.). *Heredity* 111: 495–504.

Linhart, Y. B., and J. D. Thompson. 1999. Thyme is of the essence: biochemical polymorphism and multi-species deterrence. *Evolutionary Ecology Research* 1: 151–171.

Machingura, M., E. Salomon, J. M. Jez, and S. D. Ebbs. 2016. The *β*-cyanoalanine synthase pathway: beyond cyanide detoxification. *Plant, Cell & Environment* 39: 2329–2341.

Maxwell, K., and G. N. Johnson. 2000. Chlorophyll fluorescence—a practical guide. *Journal of Experimental Botany* 51: 659–668.

McDonald, A. E. 2008. Alternative oxidase: an inter-kingdom perspective on the function and regulation of this broadly distributed "cyanide-resistant" terminal oxidase. *Functional Plant Biology* 35: 535.

Melville, J., and B. Doak. 1940. Cyanogenesis in white clover. II. Isolation of glucoside constituents. *New Zealand Journal of Science and Technology, B* 22: 67–70.

Miller, J. M., and E. E. Conn. 1980. Metabolism of hydrogen cyanide by higher plants. *Plant Physiology* 65: 1199–1202.

Mizutani, F., R. Hirota, S. Amano, A. Hino, and K. Kadoya. 1991. Changes in cyanogenic glycoside content and β-cyanoalanine synthase activity in flesh and seeds of Japanese plum (*Prunus salicina* Lindl.) during development. *Journal of the Japanese Society for Horticultural Science* 59: 863–867.

Olsen, K. M., S.-C. Hsu, and L. L. Small. 2008. Evidence on the molecular basis of the *Ac/ac* adaptive cyanogenesis polymorphism in white clover (*Trifolium repens* L.). *Genetics* 179: 517–526.

Olsen, K. M., N. J. Kooyers, and L. L. Small. 2013. Recurrent gene deletions and the evolution of adaptive cyanogenesis polymorphisms in white clover (*Trifolium repens* L.). *Molecular Ecology* 22: 724–738.

Olsen, K. M., and L. L. Small. 2018. Micro- and macroevolutionary adaptation through repeated loss of a complete metabolic pathway. *New Phytologist* 219: 757–766.

Olsen, K. M., B. L. Sutherland, and L. L. Small. 2007. Molecular evolution of the *Li/li* chemical defence polymorphism in white clover (*Trifolium repens* L.). *Molecular Ecology* 16: 4180–4193.

Olsen, K. M., and M. C. Ungerer. 2008. Freezing tolerance and cyanogenesis in white clover (*Trifolium repens* L. Fabaceae). *International Journal of Plant Sciences* 169: 1141–1147.

Peiser, G. D., T.-T. Wang, N. E. Hoffman, S. F. Yang, H. Liu, and C. T. Walsh. 1984. Formation of cyanide from carbon 1 of 1-aminocyclopropane-1-carboxylic acid during its conversion to ethylene. *Proceedings of the National Academy of Sciences, USA* 81: 3059–3063.

Piotrowski, M., S. Schönfelder, and E. W. Weiler. 2001. The *Arabidopsis thaliana* isogene *NIT4* and its orthologs in tobacco encode β-cyano-l-alanine hydratase/nitrilase. *Journal of Biological Chemistry* 276: 2616–2621.

Piotrowski, M., and J. J. Volmer. 2006. Cyanide metabolism in higher plants: cyanoalanine hydratase is a *NIT4* homolog. *Plant Molecular Biology* 61: 111–122.

Poulton, J. E. 1990. Cyanogenesis in plants. *Plant Physiology* 94: 401–405.

Reznick, D., and J. Travis. 1996. The empirical study of adaptation in natural populations. *In* M. R. Rose and G. V. Lauder [eds.], Adaptation, 243–289. Academic Press, San Diego, CA, USA.

Silvertown, J., and D. Charlesworth. 2001. Introduction to plant population biology, 4th ed. Wiley-Blackwell, Oxford, UK.

Taiz, L., E. Zeiger, I. M. Møller, and A. Murphy. 2015. Plant physiology and development, 6th ed. Sinauer, NY, NY, USA.

Thompson, K. A., and M. T. J. Johnson. 2016. Antiherbivore defenses alter natural selection on plant reproductive traits. *Evolution* 70: 796–810.

Thompson, K. A., M. Renaudin, and M. T. J. Johnson. 2016. Urbanization drives the evolution of parallel clines in plant populations. *Proceedings of the Royal Society, B, Biological Sciences* 283: 20162180.

Vanlerberghe, G. 2013. Alternative oxidase: A mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. *International Journal of Molecular Sciences* 14: 6805–6847.

Vanlerberghe, G. C., A. E. Vanlerberghe, and L. McIntosh. 1997. Molecular genetic evidence of the ability of alternative oxidase to support respiratory carbon metabolism. *Plant Physiology* 113: 657–661.

Ware, W. 1925. Experiments and observations on forms and strains of *Trifolium repens* L. *Journal of Agricultural Science* 15: 47–67.

Wright, S. J., D. Cui Zhou, A. Kuhle, and K. M. Olsen. 2017. Continent-wide climatic variation drives local adaptation in North American white clover. *Journal of Heredity* 109: 78–89.

Yu, X. Z. 2015. Uptake, assimilation and toxicity of cyanogenic compounds in plants: facts and fiction. *International Journal of Environmental Science and Technology* 12: 763–774.

Zhen, Y., P. Dhakal, and M. C. Ungerer. 2011. Fitness benefits and costs of cold acclimation in *Arabidopsis thaliana*. *American Naturalist* 178: 44–52.

Zhen, Y., and M. C. Ungerer. 2008. Clinal variation in freezing tolerance among natural accessions of *Arabidopsis thaliana*. *New Phytologist* 177: 419–427.