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FREEZING TOLERANCE AND CYANOGENESIS IN WHITE CLOVER (*TRIFOLIUM REPENS* L. FABACEAE)

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White clover is naturally polymorphic for cyanogenesis, and the ecological genetics of this polymorphism has been studied for more than 60 yr. Temperature-associated cyanogenesis clines occur in both native and introduced populations. Whereas the selective factors favoring cyanide-producing plants in warm climates are well established (differential protection against generalist herbivores), those favoring acyanogenic plants in colder regions are less well understood. We tested the hypothesis originally proposed to explain this pattern: that cyanogenic plants suffer increased tissue death following freezing as a result of frost-induced cyanide autotoxicity. Tissue damage was assessed in laboratory freeze treatments at three temperatures: −10°, −11.5°, and −13°C. We examined cyanogenic and acyanogenic genotypes sampled from across the species range, as well as from polymorphic populations sampled in North Carolina. Cyanogenic genotypes exhibited lower freezing tolerance in the species-wide sample but showed no similar trend in comparisons of plants from polymorphic populations; statistical power was reduced in the latter analysis and may be a contributing factor. These results indicate that expanded genotype sampling of polymorphic populations is warranted, and they tentatively suggest that factors such as herbivore abundance and resource allocation trade-offs may contribute to the occurrence of cyanogenesis clines in white clover.

Keywords: balanced polymorphism, freezing tolerance, cline, cyanogenesis, *Trifolium repens*, white clover.

Introduction

White clover (*Trifolium repens* L. Fabaceae) is a common perennial of fields, lawns, and pastures of temperate regions worldwide (Gibson and Cope 1985). This native Eurasian species is insect pollinated and obligately outcrossing, with vegetative propagation occurring by stolons. White clover is naturally polymorphic for cyanogenesis (cyanide release upon tissue damage). The polymorphism was first noted more than 90 yr ago (Armstrong et al. 1913; Ware 1925) and has since been the subject of extensive ecological genetic research involving both natural and experimental populations. More than 50 studies on white clover cyanogenesis have been published during the past half-century (reviewed in Hughes 1991; Hayden and Parker 2002; Olsen et al. 2007, 2008). Populations of white clover show clinal variation in cyanogenesis, with frequencies of cyanide-producing plants closely correlated with minimum winter temperatures. Altitudinal and latitudinal cyanogenesis clines occur not only in the native Eurasian species range (Daday 1954*a*, 1954*b*; De Araujo 1976; Till-Bottraud et al. 1988; Pederson et al. 1996; Majumdar et al. 2004) but also in the nonnative range (Daday 1958; Fraser 1986; Ganders 1990), where populations have become established only since European colonization. This rapid evolution of cyanogenesis clines in the introduced species range suggests that the selective pressures maintaining the cyanogenesis polymorphism are strong and pervasive.

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The white clover cyanogenesis polymorphism is controlled by two independently segregating Mendelian genes: *Ac/ac* controls the presence/absence of cyanogenic glucosides (linamarin and lotaustralin) in the vacuoles of leaf and stem cells; *Li/li* controls the presence/absence of their hydrolyzing enzyme, linamarase, in the cell wall (Coop 1940; Melville and Doak 1940; Corkill 1942; reviewed in Hughes 1991). Tissue damage that causes cell rupture brings the two components together and generates the cyanogenic response. Thus, both compounds must be present for a plant to be cyanogenic, and the occurrence of two nonfunctional alleles at either locus confers the acyanogenic phenotype. Plants can be easily scored for the presence/absence of either linamarase or cyanogenic glucosides in HCN liberation assays (see "Material and Methods"). The molecular genetic bases of the *Li/li* and *Ac/ac* polymorphisms have recently been resolved; both biochemical polymorphisms arise through gene presence/absence polymorphisms (Olsen et al. 2007, 2008).

The ecological factors that favor cyanogenic white clover plants are well documented. Field observations and manipulative experiments have demonstrated that cyanogenesis in white clover serves as a deterrent against herbivores, specifically against small, grazing generalists. This has been shown in diverse studies that have included mollusks, insects, and voles (Dirzo and Harper 1982*a*, 1982*b*; Pederson and Brink 1998; Saucy et al. 1999; Viette et al. 2000). For example, in choice experiments, both slugs and voles avoid eating cyanogenic plants; in no-choice experiments where cyanogenic plants are the only available food source, these herbivores reduce overall food consumption (Dirzo and Harper 1982*a*; Saucy et al. 1999). In mixed plantings, cyanogenic plants show greater seedling survivorship than do

acyanogenic morphs as a consequence of reduced herbivore damage (Ennos 1981; Dirzo and Harper 1982*b*).

Whereas the selective factors favoring cyanogenic morphs are fairly well understood, those favoring acyanogenic plants in colder climates have remained less clear. Two main hypotheses have been proposed, one focusing on abiotic stress and the other focusing on biotic interactions. In his classic studies documenting cyanogenesis clines, Daday (1965) proposed that cyanogenic plants may suffer decreased freezing tolerance because cell rupture from freezing could lead to autotoxicity and tissue death following HCN release. Cyanide is a potent and irreversible inhibitor of cellular respiration; thus, its liberation within leaf tissue would be expected to cause a major disruption of normal physiological processes and potentially tissue death. This abiotic stress hypothesis is supported by observations of differential frost damage on cyanogenic white clover plants in the field (Daday 1965; Dirzo and Harper 1982*b*).

As an alternative to the abiotic stress hypothesis, it has also been proposed that herbivore abundance and resource allocation trade-offs may play an important role in the evolution of cyanogenesis clines. If generalist herbivores of white clover are less abundant in cooler climates, then plants investing in growth rather than cyanogenesis may be at a competitive advantage under these conditions (Kakes 1987). Support for this herbivore-release hypothesis comes from observations of trade-offs between cyanogenesis and several fitness-related traits, including flowering rate (Daday 1965; Foulds and Grime 1972; Dirzo and Harper 1982*b*; Kakes 1987), drought tolerance (Foulds and Grime 1972), and resistance to rust (Dirzo and Harper 1982*b*).

Of these two alternatives, the freezing tolerance hypothesis is most conducive to experimental testing, as testing the herbivore-release hypothesis would require documenting worldwide temperature-associated gradients in clover herbivores. Surprisingly, there do not appear to be any published studies that have tested this abiotic stress hypothesis in white clover under controlled experimental conditions. The purpose of this study was to test whether cyanogenic white clover plants show a decrease in freezing tolerance relative to acyanogenic plants in laboratory freezing experiments. This study specifically examined the abiotic stress hypothesis as originally envisioned by Daday (1965). We compared cyanogenic and acyanogenic plants for differences in freezing-induced tissue death. Other potential temperature-associated physiological differences between cyanogenic and acyanogenic plants (e.g., rates of cellular respiration) were not assessed here.

Material and Methods

Sampling and Cyanogenesis Assays

Freezing experiments were performed using two sample sets. In the first, cyanogenic and acyanogenic plants were selected from the USDA white clover germplasm collection (representing a worldwide sample of wild *Trifolium repens* populations), with a sampling scheme designed to maximize the geographical overlap of cyanogenic and acyanogenic plants used in assays of freezing tolerance. Because microclimatic differences can vary widely over short geographical distances, this sampling scheme should be considered a crude correction for local climatic adaptation. In the second sample set, pairs of cyanogenic and acyanogenic plants were collected from populations polymorphic for cyanogenesis, with all sampled populations occurring in a localized geographical region (western North Carolina). This second sampling approach minimized any potential confounding effects of differences in local climatic adaptation between cyanotypes.

Thirty white clover accessions (15 cyanogenic, 15 acyanogenic) were selected for freezing tolerance assays (table 1). Eighteen of the accessions (nine cyanogenic, nine acyanogenic) come from the USDA germplasm collection and represent wild plants sampled from across the species range. Twelve accessions represent pairs of cyanogenic and acyanogenic plants collected from six polymorphic populations in western North Carolina (table 1). All of the USDA accessions have been previously tested for cyanogenesis, using a semiquantitative HCN assay; the cyanogenic samples all have cyanogenesis scores of >90 on a scale of 0–100, indicating that all are strongly cyanogenic, while the acyanogenic plants all have scores of 0 (http://www.ars-grin.gov).

To confirm cyanotypes of USDA samples and to test North Carolina accessions for cyanogenesis, we employed a modified Feigl-Anger HCN assay (Feigl and Anger 1966), using the protocol described by Olsen et al. (2007). Through the exogenous addition of either linamarin or linamarase to a leaf sample, it is possible to infer whether an acyanogenic plant possesses either of the underlying cyanogenesis components (i.e., functional alleles at either *Ac* or *Li*). Unless otherwise noted in table 1, all acyanogenic plants used in this study lack both components required for the cyanogenic response. Both *Ac* and *Li* show incomplete dominance at the biochemical level (reviewed in Hughes 1991). The Feigl-Anger assay is semiquantitative and can be used to distinguish weakly cyanogenic samples from moderately or strongly cyanogenic samples. Our HCN assays of cyanogenic USDA accessions confirmed that all are strongly cyanogenic. Similarly, all cyanogenic North Carolina accessions that were selected for the study showed cyanogenic responses comparable to those of the USDA accessions. Thus, both the USDA and the North Carolina cyanogenic accessions are likely to be homozygous for functional alleles at both loci.

Eight clonal replicates of each accession were used per freezing treatment for each of three different freezing treatments. Stem cuttings were taken three times over a 3-wk period from greenhouse-grown plants, and all plants were allowed to become established for at least 1 mo before freeze treatments. Plants from each of the three stem-cutting cohorts were distributed equally among the three freeze treatments to minimize any potential effects of plant maturity on freeze tolerance; individuals from the different cohorts were visually indistinguishable by 1 mo after the last cuttings were taken. Cuttings were rooted on a mist bench in the greenhouses of Washington University in St. Louis; plants were grown in flats of 3-in pots containing soil dusted with rhizobial inoculum. As stem cuttings became established, stolons were pinched to encourage bushy growth and to prevent growth into neighboring pots. Plants were grown under uniform greenhouse conditions at Washington University in the spring of 2006. Before the freeze tests, plants were cold-acclimated for 1 wk (4°C, continuous lighting) at Kansas State University.

Table 1

White Clover Accessions Used in Freezing Tolerance Assays

Accession	Cyanogenic	Latitude (°N)	Origin
PI 205062	No[a]	40.733	Tifi, Unye, Ordu, Turkey
PI 234678	Yes	43.570	Near Montpellier, Herault, France
PI 239977	Yes	38.583	Aguas de Moura, Portugal
PI 251053	No	41.66	Lake Mavrovo,[b] Macedonia
PI 251197	No	43.850	Romanian Mountains, east of Sarajevo, Bosnia/Herzegovina
PI 291828	Yes	na	Chile
PI 294546	Yes	na	French Alps
PI 298485	Yes	33.210	Kiryat Shemona, Israel
PI 319139	Yes	38.717	Almodabar del Camp, Ciudad Real, Spain
PI 384699	Yes	31.633	Near Tisi-n-text, Morocco
PI 418905	Yes	41.570	Near Isernia, Molise, Italy
PI 418914	No	44.167	Near Petramela, Italy
PI 420001	Yes	na	Japan
PI 440746	No[a]	44.790	Mount Nedryemanya, south of Stavropol, Russia
PI 494747	No	44.417	Near Domnesti, Romania
PI 499685	No[a]	44.100	Near Tian Lake, Xinjiang, China
PI 516408	No	~1.0	Near Kitale, Kenya
PI 542904	No	44.850	Near Licki, Croatia
NC01_02	No	35.570	Denver, North Carolina
NC01_09	Yes	35.570	Denver, North Carolina
NC02_02	No	35.920	Brookford, North Carolina
NC02_08	Yes	35.920	Brookford, North Carolina
NC03_07	No	35.850	Drexel, North Carolina
NC03_06	Yes	35.850	Drexel, North Carolina
NC11_08	No	35.730	Highway 128, near Mount Mitchell State Park entrance
NC11_04	Yes	35.730	Highway 128, near Mount Mitchell State Park entrance
NC13_01	No	35.770	Mount Mitchell State Park
NC13_11	Yes	35.770	Mount Mitchell State Park
NC15_06	Yes	35.750	Near Balsam Gap Overlook, Blue Ridge Parkway
NC15_11	No	35.750	Near Balsam Gap Overlook, Blue Ridge Parkway

Note. Unless otherwise noted, all acyanogenic plants lack both biochemical components of the cyanogenesis response.

[a] Cyanogenic glucosides present but lacking linamarase.

[b] Recorded as Mavrosko Lake in original collection data (http://www.ars-grin.gov/npgs/index.html).

Freezing Tolerance Assays

Assays of freezing tolerance were conducted under conditions of darkness in an ESPEC (Hudsonville, MI) ESU-3CA Platinous series environmental test chamber. On the basis of preliminary trials, three minimum temperatures were selected for assaying freezing damage (−10°, −11.5°, and −13°C). These temperatures are similar to those used in other studies of freezing tolerance in white clover (Annicchiarico et al. 2001). Plants were subjected to these minimum temperatures for 2.5 h and experienced rates of temperature change of 2°C/h during cooling and warming periods. To prevent supercooling of plant tissue during these assays, ice chips were added to all pots when the chamber temperature reached −1°C. Plant positions were randomized within the chamber during all trials. After the freezing trials, plants were allowed to recover at 4°C for 24 h and were then returned to greenhouse conditions that were identical to pretreatment conditions. After 5 d (by which time necrotic tissue was clearly identifiable), plants were scored for aboveground (leaf and stem) tissue damage using the following scale: 0, 100% tissue death; 1, >75% but <100% tissue death; 2, >50% but ≤75% tissue death; 3, >25% but ≤50% tissue death; and 4, <25% tissue death. All scoring of tissue damage was performed blind with respect to plant cyanotype.

Statistical Analysis

All data were analyzed by mixed-model ANOVA. Because of differences in data set structure between the worldwide accessions and the North Carolina populations, different statistical models were employed for the two experimental data sets. The worldwide accession data were analyzed according to the model

$$y = \mu + A(C) + T + C + A(C) \times T + T \times C + E,$$

where A represents accession of *T. repens* (random effect, nested within cyanotype), C represents cyanotype, T represents temperature, and E represents residual error. The North Carolina data set was analyzed according the model

$$y = \mu + P + T + C + P \times T + P \times C + T \times C + E,$$

where P represents population of *T. repens* from North Carolina and all other variables are as described earlier. All statis-

Table 2

Mixed-Model ANOVA Results from Freezing Tolerance Assays of Worldwide *Trifolium repens* Accessions

Source	df	SS	MS	*F*	*P*
Accession (cyanotype)	16	33.1132	2.06957	1.5932	.1279
Temperature	2	13.5591	6.77954	5.2194	.0109
Cyanotype	1	9.76362	9.76362	4.7209	.0451
Accession (cyanotype) × temperature	32	41.5725	1.29914	1.0642	.3768
Temperature × cyanotype	2	.26309	.13154	.1013	.9040
Error	378	461.4377	1.22073		

Note. Significant effects are underlined. SS = sum of squared deviations; MS = mean of squared deviations.

tical analyses were conducted using the software package JMP IN 5.1 (Sall et al. 2001).

Results

For the worldwide (USDA) *Trifolium repens* accessions, results from the full ANOVA model indicate significant effects of both temperature and cyanotype (table 2). Freezing tolerance decreased with decreasing temperature in the three freezing treatments, as expected (fig. 1). This pattern is evident even across the relatively small temperature increments of −1.5°C, an indication that the selected temperatures are appropriate for assessing freezing-induced tissue damage. The effect of cyanotype is significant in the ANOVA model ($P = 0.045$), with a pattern of response consistent with the hypothesis that cyanogenic plants suffer increased freezing damage relative to their acyanogenic counterparts (fig. 1). This difference in cold tolerance persists across all temperatures assayed, with cyanogenic plants exhibiting freezing tolerance scores of 1.87 ± 0.14, 1.70 ± 0.14, and 1.49 ± 0.13 at −10°, −11.5°, and −13°C, respectively, and acyanogenic plants exhibiting freezing tolerance scores of 2.23 ± 0.14, 1.98 ± 0.13, and 1.74 ± 0.13 at those same temperatures.

Identical freeze-treatment assays on North Carolina populations yielded results that contrasted with those of the worldwide accessions. Although a significant effect of temperature was detected (table 3; fig. 2), assays of freezing tolerance on the North Carolina plants did not reveal a significant effect of cyanotype, nor was there evidence of a trend across temperature treatments indicating decreased freezing tolerance among cyanogenic plants (fig. 2). For the two lowest temperatures, there was a weak, statistically nonsignificant trend in the opposite direction, with slightly differentially lower freezing tolerance scores for acyanogenic plants. Cyanogenic plants exhibited freezing tolerance scores of 2.64 ± 0.15, 2.09 ± 0.16, and 2.12 ± 0.16 at −10°, −11.5°, and −13°C, respectively, whereas acyanogenic plants exhibited freezing tolerance scores of 2.68 ± 0.16, 1.88 ± 0.16, and 1.76 ± 0.15 at those same temperatures. The overall mean freezing tolerance score across treatments and cyanotypes for North Carolina plants (2.19, SD = 1.13) was similar to that observed for the worldwide sample set (1.83, SD = 1.14), an indication that the two sample sets are roughly comparable. However, because of the different sizes of the worldwide and North Carolina data sets, the statistical power to detect an effect of cyanotype was considerably higher in the former (for $\alpha = 0.05$, the power to detect an effect of cyanotype [$1 - \beta$] was 0.78 and 0.28 for the worldwide and North Carolina data sets, respectively).

Discussion

The white clover cyanogenesis polymorphism has been studied for nearly a century (Armstrong et al. 1913) and is cited as a textbook example of adaptive variation maintained by opposing selective pressures (Dirzo and Sarukhan 1984; Silvertown and Charlesworth 2001). Of the two main explanations that have been proposed to account for the differential fitness of acyanogenic plants in colder climates (differential freezing tolerance and resource allocation trade-offs in the absence of herbivores), this study was designed to test the former hypothesis. We examined whether cyanogenic genotypes exhibited lower freezing tolerance, measured as aboveground tissue death, when exposed to temperatures of −10°, −11.5°, and −13°C. Because differences in freezing tolerance also may evolve through local adaptation to different climatic conditions, we examined cyanogenic and acyanogenic genotypes from overlapping regions across the species range as well as from in-

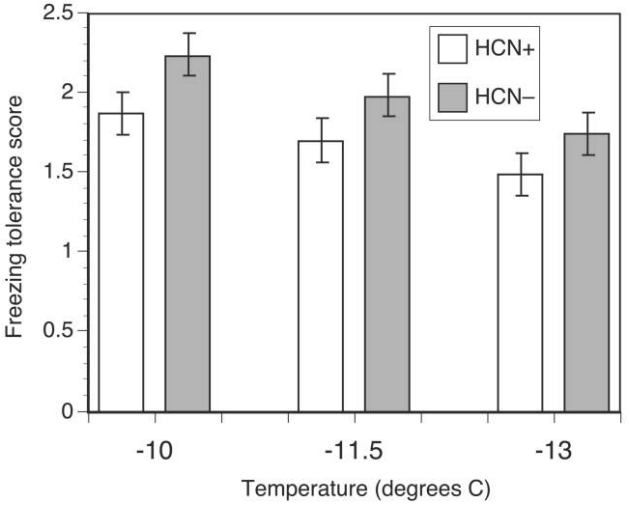

Fig. 1 Least square means (with SE) of freezing tolerance scores for worldwide *Trifolium repens* accessions grouped by cyanotype. Freezing tolerance assays were conducted at three different minimum temperatures. HCN+ and HCN− refer to cyanogenic and acyanogenic plants, respectively.

Table 3

Mixed-Model ANOVA Results from Freezing Tolerance Assays of North Carolina *Trifolium repens* Populations

Source	df	SS	MS	*F*	*P*
Population	5	14.7946	2.95892	1.4130	.3712
Temperature	2	30.9837	15.4918	13.6539	.0014
Cyanotype	1	2.18104	2.18104	1.0419	.3542
Population × temperature	10	11.3461	1.13461	1.0004	.4435
Population × cyanotype	5	10.4663	2.09326	1.8456	.1044
Temperature × cyanotype	2	2.07526	1.03763	.9149	.4019
Error	260	294.889	1.13419		

Note. Significant effects are underlined. SS = sum of squared deviations; MS = mean of squared deviations.

troduced polymorphic populations in North America (North Carolina). If cyanogenesis per se were a major contributor to decreased freezing tolerance, cyanogenic plants in any given population should have decreased freezing tolerance relative to their acyanogenic neighbors. Interestingly, we did not observe consistent patterns of decreased freezing tolerance among cyanogenic genotypes. Cyanogenic genotypes exhibited lower freezing tolerance than acyanogenic genotypes in the collection of accessions from across the species range (table 2; fig. 1) but did not show lower freezing tolerances in identical assays comparing cyanogenic and acyanogenic genotypes from polymorphic North Carolina populations (table 3; fig. 2). Because plants from the two experimental groups were assayed simultaneously and their positions were randomized during bouts of freezing stress, our results are not readily attributable to differences in freezing treatments.

The failure to detect consistent patterns of decreased freezing tolerance in cyanogenic genotypes suggests that either (1) statistical power to detect an effect of cyanotype was lacking in our analyses, given the experimental methods, or (2) cyanogenesis alone may not be a major contributor to decreased freezing tolerance, and differences between cyanotypes sampled from across the species range (fig. 1) may be attributable to local climate adaptation, with cyanogenesis representing a correlated response. We discuss both of these possibilities.

The total number of genotypes examined here (30 genotypes, eight clonal replicates per genotype) is clearly a small subset of the full genetic variation present in *Trifolium repens*. It is possible that expanding the sample size could reveal associations between cyanogenesis and freezing tolerance that are not detectable with this sample size; this potential limitation of this study may be especially relevant for the analyses of polymorphic North Carolina populations, where statistical power is lowest and where no statistically significant effect of cyanotype was detected (table 3). Although the sample sizes used in this study are admittedly limited, it is worth noting that the sampling is sufficient to reveal statistically significant differences between the two cyanotypes in the USDA sample set (table 2), as well as among the three temperature treatments in both the USDA and the North Carolina sample sets (tables 2, 3). Moreover, for the two lowest-temperature treatments of the North Carolina data set, the observed trend, although statistically nonsignificant, was in the opposite direction of that predicted by the freezing tolerance hypothesis (fig. 2). Additional insights might very well be gained by readdressing this question, using an expanded set of genotypes, and by focusing on polymorphic populations where effects of local climatic adaptation do not come into play.

A second potential limitation of our experimental design involves the methods used in assessing freezing tolerance. We have examined fitness specifically as aboveground tissue death after freezing. It is possible that there are more subtle cold-associated fitness differences between cyanogenic and acyanogenic plants that would not have been detected in our analyses. Cellular respiration rate is one potential factor. Brighton and Horne (1977) compared leaf oxygen consumption after freezing in cyanogenic and acyanogenic plants of a different species, *Lotus corniculatus*; they found that freezing differentially impaired levels of leaf respiration in cyanogenic plants, with effects lasting at least 48 h after the freeze treatment. It should be noted, however, that their study reported not only differential respiration rates but also differential tissue death in the cyanogenic plants. Thus, assessing fitness by measuring tissue dieback would presumably have led to similar conclusions as would have measuring oxygen consumption (albeit not necessarily with the same degree of statistical significance). It should also be noted that in the analysis by

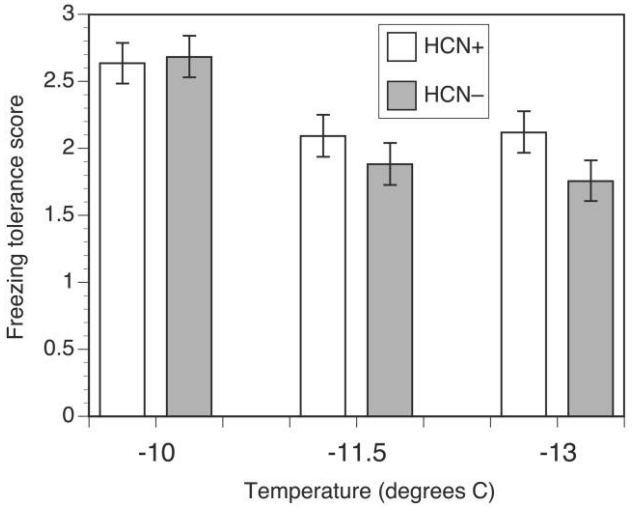

Fig. 2 Least square means (with SE) of freezing tolerance scores for North Carolina populations of *Trifolium repens* grouped by cyanotype. Freezing tolerance assays were conducted at three different minimum temperatures. HCN+ and HCN− refer to cyanogenic and acyanogenic plants, respectively.

Brighton and Horne (1977), cyanogenic and acyanogenic *L. corniculatus* plants were sampled from along a sharp elevational cline; thus, it is unclear whether the observed association with respiration rate reflects cyanogenesis per se or other factors related to cold adaptation.

Potential limitations of experimental methodology notwithstanding, our study tentatively suggests that cyanogenesis may not be a major determinant of freezing tolerance in white clover. If this pattern holds with expanded genotype sampling, it would have significant implications for understanding the evolutionary dynamics of this long-studied balanced polymorphism. Previous evidence for the freezing tolerance hypothesis has been based primarily on field observations of differential frost-induced tissue damage in cyanogenic plants (Daday 1965; Dirzo and Harper 1982*b*). In field experiments comparing a single cyanogenic and acyanogenic genotype grown in an alpine environment, Daday (1965) observed statistically significantly greater frost damage on the cyanogenic genotype. Citing unpublished observations, Daday (1965, p. 362) also reported that cold-induced HCN liberation "caused an irreversible inhibition of the respiratory system of the *AcLi* genotype, resulting in tissue death." In a later study, Dirzo and Harper (1982*b*) reported a higher intensity of frost damage on cyanogenic plants in the early part of the winter; however, they observed the opposite pattern later in the winter, so that the overall effect was not significant.

The main alternative to the abiotic stress hypothesis is that the abundance of clover herbivores declines in colder climates and that, in the absence of these herbivores, plants investing in growth and reproduction rather than defense are at a competitive advantage. Definitive proof for this hypothesis would require demonstrating (1) worldwide correlations between winter temperatures and the abundance of generalist clover herbivores and (2) trade-offs between cyanogenesis and growth/reproduction. Although demonstrating the first factor is not an easy undertaking, demonstrating the second is fairly straightforward and has already been addressed in a number of studies (reviewed in Hayden and Parker 2002). These studies have documented trade-offs between cyanogenesis and several fitness-related traits, including flowering rate (Daday 1965; Foulds and Grime 1972; Dirzo and Harper 1982*b*; Kakes 1989), resistance to rust (Dirzo and Harper 1982*b*), and drought tolerance (Foulds and Grime 1972).

Beyond these direct observations of cyanogenesis-related fitness trade-offs, additional evidence of cyanogenesis-related costs comes from data on the biochemical and genetic basis of the polymorphism. Linamarase, the enzyme required for the cyanogenic response, constitutes ~5% of the soluble leaf protein in cyanogenic plants (Hughes and Dunn 1982). Synthesis of such a large amount of any protein represents a very large energetic investment; this would be especially true for linamarase, as the enzyme has no known function other than in cyanogenesis (see Barrett et al. 1995). The energetic costs of cyanogenesis probably account for the relative rarity in natural populations of plants that possess only one of the two required components for functional cyanogenesis; although *Ac*/*ac* and *Li*/*li* are independently segregating genes, functional alleles at the two genes are in strong linkage disequilibrium (Ennos 1982). Plants with *acLi* or *Acli* genotypes (possessing only linamarase or only cyanogenic glucosides, respectively) are presumably selected against because they carry some of the energetic costs of cyanogenesis without the benefits. The observation of strong linkage disequilibrium between these independently segregating loci cannot easily be explained by the abiotic stress (freezing tolerance) hypothesis alone because the acyanogenic phenotype does not require the simultaneous occurrence of nonfunctional alleles at both genes.

In conclusion, we find limited evidence for the hypothesis that cyanogenic plants suffer increased tissue damage resulting from exposure to freezing temperatures. Our failure to consistently detect this pattern across our two data sets suggests that this phenomenon, if real, may not be as overwhelmingly strong as has been previously suggested (Brighton and Horne 1977) and may likely be one of several factors influencing the biogeography of this well-characterized polymorphism. We also note that while biotic and abiotic stresses are discussed here as two alternative explanations for cyanogenesis clines, the two mechanisms need not be mutually exclusive. Expanded sample sizes, field observations of herbivore abundance/impact, and ecophysiological assays may all ultimately be required to definitively determine the ecological factors underlying this balanced polymorphism.

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