

## Plasticity in cyanogenesis of *Trifolium repens* L.: inducibility, fitness costs and variable expression

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### ABSTRACT

The polymorphism for cyanogenesis – the production of cyanide by damaged tissue – in white clover (*Trifolium repens* L.) has long been a model system for studying the maintenance of genetic variability. The prevailing model holds that opposing selective forces maintain the polymorphism; cyanogenesis protects the clover from herbivory, but incurs a cost of defence at lower temperatures and under drought. To date, most studies of cyanogenesis have focused on the presence or absence of the trait, although there is evidence of variability in expression within individuals. It is known that inducibility in some plant defence systems alleviates costs by initiating or increasing the expression of a costly defence only when it is most needed. It follows, then, that defences might also be downregulated when they are likely to be most costly. We used a modified Feigl-Anger assay to quantify the extent of cyanogenesis (both cyanoglucoside and  $\beta$ -glucosidase enzyme) in clones of *T. repens* under different environmental conditions, historically associated with the costs and benefits of the polymorphism. Neither simulated herbivory nor herbivory by the snail *Helix aspersa* resulted in a significant increase in expression of cyanogenesis (cyanoglucoside or enzyme). Therefore, we conclude that cyanogenesis is not an inducible defence. However, drought stress led to an apparent decrease in activity of  $\beta$ -glucosidase in samples with amplified linamarin concentrations. Furthermore, genotypes showed significantly weaker expression when grown in cold than when grown in warm temperatures. Our results suggest that some conditions that favour acyanogenic plants may also result in a decreased expression in cyanogenic morphs – a plasticity that changes our understanding of the selective forces at work in this system.

*Keywords:* chemistry, cost of defence, cyanogenesis, plant defence, plasticity.

### INTRODUCTION

Genetic polymorphisms have long served as model systems for the study of forces that maintain genetic diversity as a whole. Any trait providing a fitness advantage should come to dominate a population; a polymorphism, then, requires some intervening dynamic of

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forces to persist. One process that can lead to stable polymorphisms is frequency-dependent selection, as in the classic case of self-incompatibility systems in plants (Fisher, 1941, 1944), or alternate-mating strategies in animals (Sinervo and Lively, 1996). A second process leading to stable polymorphisms arises from competing selective forces acting on a fitness trade-off. Such trade-offs are implicated in the different herbivore preferences and competitive ability associated with polymorphic monoterpenes in thyme (Lamiaceae: *Thymus vulgaris*) (Linhart and Thompson, 1999), and in the polymorphism for slow-growing, taller morphs (i.e. better competitors for light) and fast-growing, short morphs (i.e. better able to recover from herbivory) in the Serengeti grass *Sporobolus kentrophyllus* (Hartvigsen and McNaughton, 1995).

Furthermore, in the face of opposing selective forces, selection ought to favour traits that lessen cost, such as phenotypic plasticity. Plasticity may act to mask a genotype from selection (Wright, 1931; Anstey, 1987), or it may even act in an explicitly adaptive manner to allow an organism to express the genotype best suited to a particular environment (Schlichting, 1986). Examples of adaptive plasticity include shade avoidance in plants (reviewed in Schmitt, 1997) and such defensive strategies as the predator-induced development of a less fecund but predator-resistant shell shape in the barnacle *Chthamalus anisopoma* (Lively, 1999). Such induced defences have received considerable attention in recent years, especially concerning plant biochemical defences. It is broadly assumed that where a chemical defence incurs a fitness cost when the enemy is absent, the ability to express an expensive defence only when the probability of attack is high provides an organism with the best of both worlds: avoiding costs of an unnecessary defence, while enjoying its benefits in the presence of an enemy (Karban and Baldwin, 1997).

The polymorphism for cyanogenesis of white clover (*Trifolium repens* L.) has long been studied as a model system. The ability to release hydrogen cyanide (HCN) after tissue damage requires the presence within the plant tissue of both a cyanoglucoside (in white clover, a mixture of lotaustralin and linamarin; Melville and Doak, 1940) and a  $\beta$ -glucosidase enzyme, linamarase (Corkill, 1940). Acyanogenic plants may lack the cyanoglucoside, the enzyme or both. A single dominant gene, Li, controls inheritance of linamarase, while production of both linamarin and lotaustralin is controlled by the dominant gene, Ac (Corkill, 1942). The production of cyanide follows two steps. First, a sugar is cleaved from the cyanoglucoside by linamarase. The resulting cyanohydrin is then hydrolysed by a hydroxynitrile lyase, releasing hydrogen cyanide (Poulton, 1990). The plant prevents premature release of cyanide – and self-poisoning – by sequestering linamarase in the cell wall, so that the enzyme and cyanoglucoside only come in contact if the cell is damaged (Kakes, 1985).

Daday's classic studies on the distribution patterns of cyanomorphs found that, both in the clover's native Eurasia and worldwide, the dominant forms of Ac and Li were more common at higher mean temperatures and at lower altitudes (Daday, 1954a,b, 1958). Later researchers have largely confirmed Daday's patterns of distribution. Till (1987) found similar patterns in France, in that white clover at higher altitude was more likely to be acyanogenic or only weakly cyanogenic. Pederson *et al.* (1996) found the temperature and altitude correlation was upheld in the United States, and Caradus and Forde (1996) confirmed the patterns in eastern Turkey. In the Netherlands, however, Kakes (1987) found that cyanomorph frequency did not correspond to altitude or temperature clines.

Daday (1965) attributed this pattern to the opposing selective forces that are generally thought to maintain the polymorphism: protection from herbivory for cyanogenic plants

counteracted by a cost in increased susceptibility to frost. Cyanogenesis' activity as a herbivore defence has been well supported; both molluscs and voles have been shown to prefer to eat acyanogenic morphs (Dirzo and Harper, 1982a; Saucy *et al.*, 1999; Viette *et al.*, 2000).

Likewise, cyanogenesis appears to incur a considerable cost. Daday (1965) observed greater frost damage in cyanogenic morphs. In Dirzo and Harper's (1982b) study, cyanogenic plants were significantly more damaged by frost and by the rust *Uromyces trifolii*, which infected very few acyanogenic plants, although other pathogens did damage both morphs. Foulds and Grime (1972) found cyanogenic morphs more susceptible to drought. Furthermore, several authors have noted lower flowering rates in cyanogenic morphs (Daday, 1965; Foulds and Grime, 1972; Dirzo and Harper, 1982b; Kakes, 1997). Interestingly, Kakes (1997) found both decreased flowering rates and protection from herbivory to be correlated only with possession of the gene *Ac*, although Dirzo and Harper (1982b) found most – but not all – of their test animals did not distinguish between *Acli* and *acli* or *acli* phenotypes.

In addition to qualitative variation in phenotype between *T. repens* plants, plants may also vary quantitatively. Based on coarse differences in perceived colour intensity on a test paper, Corkill's and Daday's classic papers note variation in the amount of cyanide produced, both between and within genotypes (Corkill, 1940; Daday, 1954b). Some of the variation across genotypes is explainable by incomplete dominance at *Ac* and *Li*; *T. repens* that are heterozygous at either locus produce an intermediate amount of the respective compound (Hughes and Stirling, 1982; Hughes and Dunn, 1985). However, variability within genotypes suggests some additional means of control over expression (Hughes *et al.*, 1988). Dirzo and Harper (1982b) noted that testing individual leaves for cyanotype gave inconsistent results. In a study of *T. repens* in France, Till (1987) also found heterogeneity within plants; indeed, she found no plants that were entirely constant for cyanotype.

Research to date on the cyanogenesis polymorphism in white clover has focused primarily on the trait as an opportunity to study the cost of defence and on searching for differences in fitness under different environmental conditions to explain the persistence of the polymorphism. Decades of work on cyanogenesis in *T. repens* have provided us with an idea of which environments should favour cyanogenic alleles and which should select against them. These same environments should also select for modifiers that increase or decrease the expression of cyanogenesis in plants that carry the alleles. Till's work in particular suggests that plasticity does exist in this system and that genotypes may differ qualitatively in their expression of cyanogenesis.

The work presented here extends the investigation of plasticity to quantitative variation and the environmental factors that lead to differential expression. If adaptive plasticity occurs in this system, cyanogenesis should be more pronounced in the presence of chronic herbivory. Similarly, cyanogenesis should be reduced in environments with cold conditions or periodic droughts. We use an experimental approach to evaluate the possibility of such plastic responses, contributing to the understanding of benefits and costs maintaining this polymorphism.

## MATERIALS AND METHODS

*Trifolium repens* is a perennial legume, native to Eurasia. It has been deliberately introduced worldwide as a forage crop and has been documented in feral populations in

Eurasia, Africa, the Americas and Australia (Daday, 1958). *T. repens* seeds from more than 15 individuals were collected in July 1998 from a roadside population at the University of California Natural Reserve at Bodega Bay, California. At this site, *T. repens* occurs most commonly in disturbed areas and in lawns and gardens, while it grows poorly and is rarely found in the native coastal prairie (I.M. Parker and G.S. Gilbert, unpublished data). Bodega Bay experiences a Mediterranean climate; lowest temperatures occur in January (average min/max 5.2°C/13.9°C), while September is the warmest month (average min/max 10.9°C/18.1°C). Average monthly rainfall varies from 0.3 cm in July to 17.4 cm in January (Chow, 2000). Several species of native and introduced *Trifolium* species co-occur within the reserve.

Seeds were individually nicked with a razor blade, then allowed to germinate in petri plates on filter paper moistened with distilled water. Once a cotyledon was visible (after 4–7 days), we transplanted seedlings to individual 4.5 cm plastic pots in Premier brand Pro-Mix PGX growth medium and grew them in a greenhouse (day/night regime of 16/8 h, temperature 21–23°C, mean relative humidity 65%). All plants, except those in the drought experiment, were fertilized 5 days per week with 100 ppm N of Peter's 20, 20, 20 liquid fertilizer, and watered with tap water on the remaining 2 days.

We used a modified Feigl-Anger assay (as described by Kakes, 1991, and outlined below) to quantify cyanogenesis in clones of *T. repens* under different environmental conditions historically associated with the costs and benefits of the polymorphic system. We first determined the repeatability of our method of testing and the impact of developmental stage on expression. We then subjected cuttings to simulated herbivory, snail herbivory, cold and drought and compared the amount of cyanide given off by leaf pieces from the treatment clones to their respective controls.

### Repeatability

We quantified the precision of the method of testing within and across plates and test papers by sampling nine leaf pieces from one first, unfolded leaf on each of four plants. Each leaf piece was randomly assigned a position in three different plates, so that each plate contained three leaf pieces from each plant. These pieces were tested for cyanogenesis (see 'Testing for cyanogenesis'), although not for cyanoglucoside and enzyme production separately. Observed cyanogenesis varied significantly among assay plates (Table 1); however, this effect was small compared with the variability among plants assayed. To correct

**Table 1.** ANOVA results for effect of assay plate and plant for four cyanogenic plants replicated within and between three 96 well plates.

Source of variation	SS	MS Num	d.f.	F	P
Plant	0.11	0.037	3	76.18	< 0.0001
Plate	0.0084	0.004	2	8.37	0.0018
Plant*Plate	0.0025	0.00042	6	0.83	0.56
Residual	0.012	0.001	24		

*Note:* Both plant and plate were treated as random effects and tested over the residual.

for variation due to test plate, we assayed paired samples from experimental and control individuals within a single plate for all the experiments reported here. There was no significant interaction of plate and plant, meaning that the magnitude of treatment/control comparisons should not vary among plates.

### Developmental variation

We tested for variation in expression within plants by developmental stage by sampling four leaves from a single stolon on four different plants, starting with the youngest, still-folded leaf, then the first unfolded, and two more leaves down the stolon. Because there was a significant effect of leaf stage, we sampled leaf pieces for assay from the youngest unfolded leaf on a stolon for all experiments.

### Simulated herbivory

To test for a response to physical damage, we simulated herbivory mechanically using a standard hole-punch (Green and Ryan, 1972). We divided 24 cyanogenic *T. repens* genets into two clonal ramets (hereafter called 'clones'): 5 cm of stolon with two unfolded leaves were weighed, dipped in rooting compound, rooted in a propagation bed and transplanted into individual 2 inch pots after 14 days. We randomly assigned each clone to either the treatment or control group and kept the clone pairs side-by-side in trays in the greenhouse, with random order of treatment and control clones. We punched a hole in each new leaf of each plant in the treatment group once weekly for 4 weeks to emulate chronic herbivory. Overall, about 500 leaves per plant were punched. After 4 weeks, plants were assayed and the data analysed for strength of cyanogenesis as described below.

### Snail herbivory

Mechanical damage does not always adequately simulate herbivory. Although Green and Ryan (1972) found similar increases in proteinase inhibitors in potato and tomato when wounded by beetles as when artificially wounded by filing or leaf-punching, other studies have found that mechanical damage does not reproduce the same response of feeding by specific herbivores (for a review, see Karban and Baldwin, 1997). Consequently, we tested cuttings of 23 cyanogenic *T. repens* for response to eating by the brown garden snail *Helix aspersa* Müller. Like *T. repens*, *H. aspersa* is native to Eurasia, but is common in lawns and gardens of the United States (Metcalf and Smartt, 1997) and has been recorded as a major consumer of white clover (Dirzo and Harper, 1982a). And like *T. repens*, *H. aspersa* is common in roadsides and cultivated areas at Bodega Bay, but is rarely found in the prairie (I.M. Parker and G.S. Gilbert, unpublished data).

Twenty-three genotypes were divided as described above, except that cuttings were not treated with rooting compound or established in propagation beds, but were placed directly into soil. Plants were kept in random order in a growth chamber (day/night regime of 14/10 h, 25°C/15°C, 60% relative humidity) to exclude herbivores. Three weeks after dividing, we randomly assigned clones to treatment and control groups and placed over each plant a 35 × 135 mm plastic cone in which a slit and a 5 × 5 mm hole had been cut. The petiole of the third unfolded leaf on one stolon was threaded through the hole. We then

allowed a snail (starved for 24 h) to feed on the treatment plants until at least 50% of the exposed leaf was removed. We marked the leaf's petiole with Liquid Paper™, then removed the cones from the clone pairs, taking care that treatment and control clones were enclosed for equivalent amounts of time. Plants were returned to the growth chamber for 5 days before assaying for cyanide production, when we tested the newest unfolded leaf on the same stolon as the marked petiole.

### Drought

Twelve cyanogenic *T. repens* L. genotypes were grown in the greenhouse. Four clones were taken from each genotype: 5 cm of stolon with two unfolded leaves was weighed (mean  $\pm$  standard deviation:  $0.28 \pm 0.06$  g), dipped in rooting compound, planted in a propagation bed and transplanted into individual 2 inch pots after 14 days. We randomly assigned each clone to drought-stressed, drought-control, cold or warm treatment groups. There were no significant differences in stolon weight among treatments (ANOVA:  $F_{3,52} = 0.72$ ,  $P = 0.97$ ).

To simulate drought stress, we allowed drought-treatment plants to wilt, then watered each plant with 50 ml of distilled water and drained surplus water. Over the 6 week trial, 10 wilting cycles were completed, with a mean of 2.9 days between waterings. Control plants were kept continuously moist with distilled water. Otherwise, both groups were maintained at the greenhouse conditions described above. Salt stress was avoided in the drought-stressed plants by liberally watering and draining plants in both groups three to five times a day for 4 days before beginning treatment, to leach salts from the growth medium.

### Temperature

The effect of growth in cold temperatures was tested in two independent experiments. The first was begun on 24 February 2000 using the same 12 parent genotypes and the cold- and warm-treatment clones as described for the drought experiment. Clones were assigned random positions in two growth chambers maintained at 14 h days, 60% relative humidity and a light intensity of 300  $\mu$ mol. During the 6 week trial, the 'cold' group was subjected to 15°C/5°C (day/night) temperatures, while the 'warm' group was kept at 25°C/15°C (day/night).

In the second temperature experiment, two clones were cut from 23 cyanogenic genotypes and the cuttings were transplanted directly into 2 inch pots. Again, there was no significant difference in stolon weight between treatments (paired *t*-test: d.f. = 36,  $P = 0.57$ ). Cuttings were allowed 21 days to root, then cold and warm treatment groups were assigned randomly. A 6 week trial was initiated with chamber conditions identical to those in the first experiment. The treatments were reversed in the growth chambers, such that the chamber programmed for cold conditions in the first experiment was programmed for warm conditions in the second. However, growth chamber malfunctions required that the 'warm' group be moved among a total of three different chambers throughout the experiment. The cold group was not moved. One malfunction resulted in one day during week 3 in which the high temperature in the warm chamber climbed to 30°C; otherwise, conditions were not noticeably affected.

### Testing for cyanogenesis

We assayed all plants before and after the treatment regimes using the modified Feigl-Anger test described by Kakes (1991). Three pieces were cut from the first unfolded leaf from one stolon of each plant to be tested, weighed to 0.00001 g on a Mettler analytical balance, then placed in a 96 well microtitre plate. Samples were frozen at  $-20^{\circ}\text{C}$  for at least 1 h, the plates were loaded as follows, then each leaf piece was macerated with a wooden pick.

Each well was loaded with 100  $\mu\text{l}$  of solution. One of the three samples taken from each plant was loaded with water only to measure the cyanogenesis due to the combined effects of the enzyme and cyanoglucoside produced by the plant. We then tested the remaining two pieces to determine whether cyanoglucoside or enzyme was limiting in the production of cyanide. We added 10  $\mu\text{l}$  of  $3.75 \text{ mmol}\cdot\text{l}^{-1}$  linamarase solution prepared from commercially obtained linamarase plus 90  $\mu\text{l}$  water to one of the two remaining leaf pieces. Then, 10  $\mu\text{l}$  of commercial snail acetone powder solution (which contains a  $\beta$ -glucosidase with the same action as linamarase, 1 U per 10  $\mu\text{l}$  aliquot) plus 90  $\mu\text{l}$  water was added to the third. Supplementing acyanogenic leaf samples with either compound is often used to determine which factor is missing – cyanoglucoside or enzyme – from a single-recessive individual, since samples with the missing factor added will test positive (e.g. Kakes, 1991). Since the colour change assay is sensitive to the strength of response, supplementing weakly cyanogenic samples with the limiting component will elicit a strong response.

On each plate, we loaded six freshly prepared standard solutions of NaCN in water, of concentrations ranging from 0.09 to  $0.63 \text{ mmol}\cdot\text{l}^{-1}$ . The plate's surface was dried, a sheet of Feigl-Anger test paper was placed over the wells, the lids were fastened with rubber bands and the plate was incubated at  $35^{\circ}\text{C}$  for 1 h. After incubation, the test papers were dried in a fume hood, then analysed.

Test papers were made by mixing equal volumes of 1% w/v solutions of tetra base (4,4'-methylenebis-(*N,N*-dimethylaniline)) and copper (II) ethylacetoacetate in chloroform. Whatman No. 2 filter papers were cut to the size of a microtitre plate, then soaked briefly (about 15 s) in the mixed solutions before being dried in a fume hood (Kakes, 1991; Seigler, 1991).

### Analysis of test papers

We used computer analysis to quantify the results of the colour change assay. Test papers were digitized within 30 min of incubation. We then obtained a numeric value for the colour saturation of the spot corresponding to each sample with NIH Image software. We constructed a standard curve for each plate by plotting the log of the mean saturation corresponding to the standard solutions against concentration. The dynamic range of the curves was empirically determined to fall below the saturation value for the  $0.64 \text{ mmol}\cdot\text{l}^{-1}$  standard for any particular plate. Samples were only included in the data set if they fell within this range. For these, we calculated the concentration of NaCN/water solution that would give off an equivalent amount of HCN for each sample from the curve, expressed here as  $\text{mmol CN}^{-}$  per mg leaf tissue. Leaf pieces of about 1 mg fresh weight usually resulted in values in the measurable range.

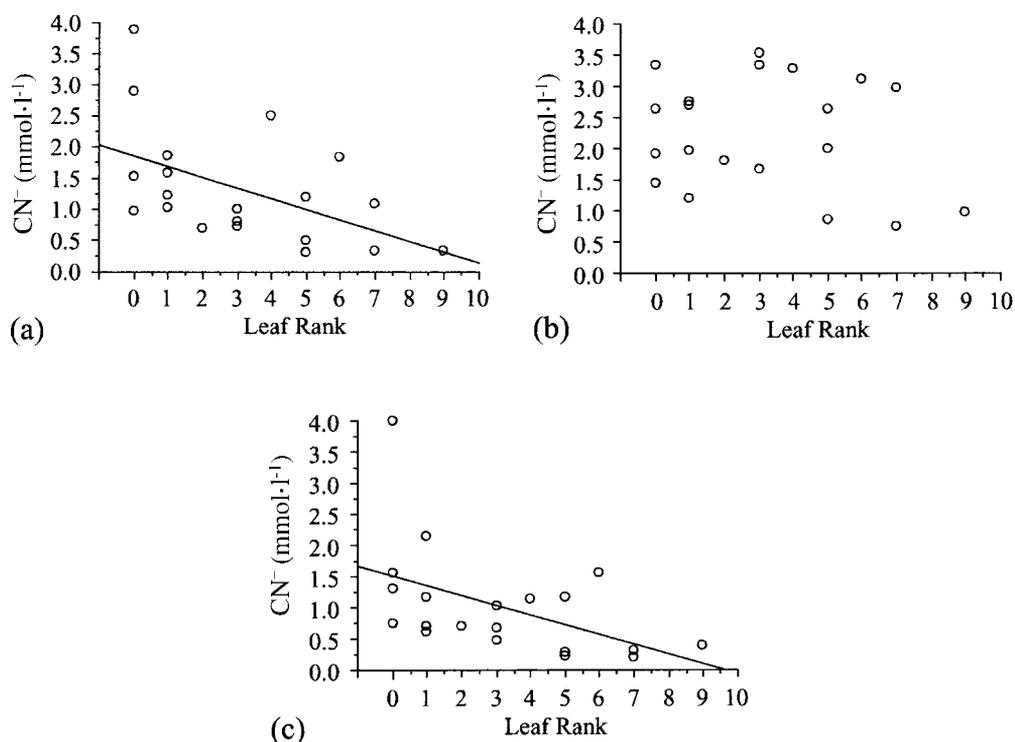
We made three comparisons per experiment – samples with water only added, samples with linamarin added and samples with enzymes added – and used one-tailed paired *t*-tests

to evaluate whether cold, drought or simulated herbivory resulted in a change in cyanogenesis relative to their respective controls. Samples with water only added represented the extent of cyanogenesis the plant was capable of by itself, and presumably that which would be experienced by a herbivore. By comparing the amount of cyanide produced in treatment versus control samples to which  $\beta$ -glucosidase or linamarin was added, we evaluated whether production or activity of cyanoglucosides or linamarase was altered, respectively.

## RESULTS

### Developmental variation

The developmental stage of the sampled leaf had a significant effect on the strength of observed cyanogenesis in unsupplemented samples and in those to which  $\beta$ -glucosidase was added (Fig. 1a,c). There was no significant effect of development stage on observed cyanogenesis in linamarin-supplemented samples (Fig. 1b). The correction of leaf stage effect in linamarin-supplemented samples suggests diminished cyanoglucoside concentration in older leaves.



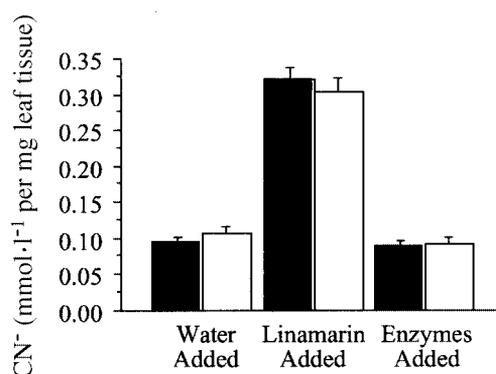
**Fig. 1.** Cyanide output versus leaf developmental stage, where leaf rank 0 is the youngest, still-folded leaf. Note that regression was performed after logarithmic transformation of the dependent variable. (a) Water added (d.f. = 1,18,  $r^2 = 0.36$ ,  $P = 0.02$ ). (b) Linamarin added (d.f. = 1,18,  $r^2 = 0.09$ ,  $P = 0.21$ ). (c) Enzymes added (d.f. = 1,18,  $r^2 = 0.38$ ,  $P = 0.04$ ).

### Simulated herbivory

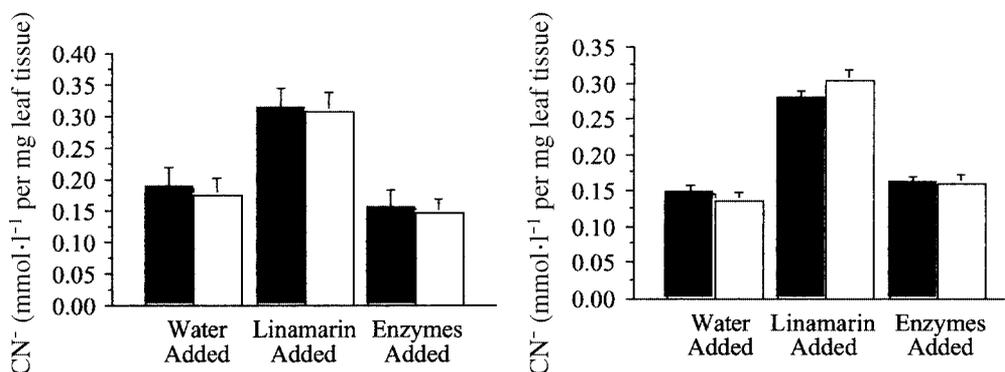
Six weeks of simulated herbivory showed no significant treatment effect on cyanogenesis relative to the control (Fig. 2). The lack of effect was consistent in all three assay groups, indicating that neither cyanoglucoside nor enzyme concentration was altered.

### Snail herbivory

There was no significant effect of snail herbivory on the newest unfolded leaf of the herbivorized stolon 5 days after treatment (Fig. 3). Again, neither cyanoglucoside nor enzyme concentration was affected.

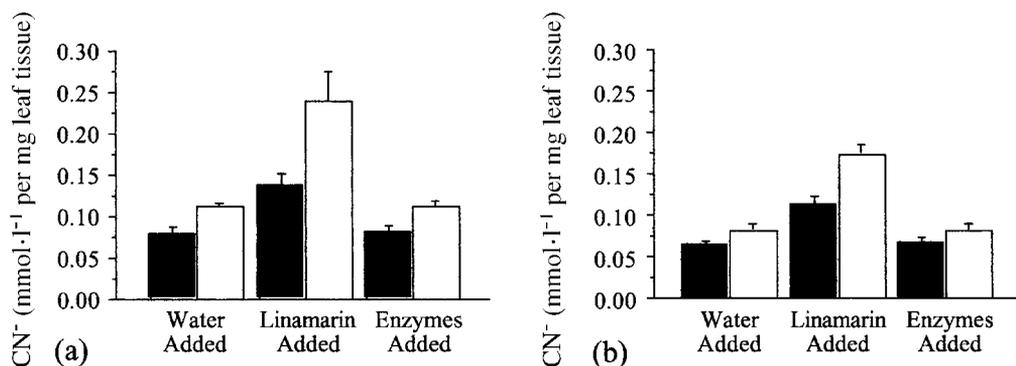


**Fig. 2.** Mean cyanide output of mechanically damaged (■) and control (□) clone pairs. Error bars represent one standard error of the mean (water added:  $t_{20} = -0.85$ ,  $P = 0.20$ ; linamarin added:  $t_{20} = -0.24$ ,  $P = 0.79$ ; enzymes added:  $t_{20} = 0.81$ ,  $P = 0.41$ ).



**Fig. 3. (Left)** Mean cyanide output of snail-herbivorized (■) and control (□) clone pairs. Error bars represent one standard error of the mean (water added:  $t_{21} = -0.58$ ,  $P = 0.57$ ; linamarin added:  $t_{21} = -0.48$ ,  $P = 0.63$ ; enzymes added:  $t_{21} = -0.25$ ,  $P = 0.81$ ).

**Fig. 4. (Right)** Mean cyanide output of drought-stressed (■) and control (□) clone pairs. Error bars represent one standard error of the mean (water added:  $t_{11} = 0.76$ ,  $P = 0.77$ ; linamarin added:  $t_{11} = -1.80$ ,  $P = 0.05$ ; enzymes added:  $t_{11} = -0.15$ ,  $P = 0.44$ ).



**Fig. 5.** Mean cyanide output of clone pairs in cold (■) and warm (□) environments. 'Cold' represents 15°C/5°C day/night temperatures; 'warm' represents 25°C/15°C day/night. Error bars represent one standard error of the mean. (a) First experiment (water added:  $t_{11} = -2.56$ ,  $P = 0.01$ ; linamarin added:  $t_9 = -2.75$ ,  $P = 0.01$ ; enzymes added:  $t_{11} = -2.73$ ,  $P = 0.01$ ). (b) Second experiment (water added:  $t_{23} = -2.49$ ,  $P = 0.02$ ; linamarin added:  $t_{23} = -4.87$ ,  $P < 0.0001$ ; enzymes added:  $t_{23} = -2.18$ ,  $P = 0.04$ ).

### Drought

Linamarin-supplemented samples revealed a significant negative effect of drought stress, indicating that drought-stressed clones were less effective at releasing HCN from cyanoglucosides (Fig. 4). Drought-stressed clones did not differ significantly from the control in unsupplemented or enzyme-supplemented leaf samples, indicating that enzyme activity, but not cyanoglucoside concentration, was reduced by drought stress.

### Temperature

Both temperature experiments showed a significant negative effect of cold temperatures on cyanogenic expression. Clones kept in cold growth chambers showed significantly lower cyanide production than those in warm chambers for all three tests: water, linamarin or enzymes added (Fig. 5a,b). Lower cyanide production in all three assays is consistent either with decreases in both cyanoglucoside content and enzyme activity, or with a decrease in cyanoglucoside content alone.

### DISCUSSION

We did not find increased cyanogenesis in response to either mechanical tissue damage or feeding by the snail *Helix aspersa*. In light of these findings, it would appear that cyanogenesis of *T. repens* is not induced in the strict sense by mollusc herbivory. Karban and Baldwin (1997) point out that induced defences may be viewed as a continuum ranging between two types, with defences whose concentrations are increased at the level of transcription at one end (induced defences in the strictest sense) and those which involve the activation of precursors already present in plant tissue (such as cyanogenesis) at the other. However, use of the second category has the potential to cloud situations in which the concentration or effectiveness of the precursors themselves is changed. For example, after wounding, white pine (*Abies grandis*) produces a different suite of resin monoterpenes from

its constitutive mixture (Steele *et al.*, 1995). Since there is variation in the amount of HCN produced by individual *T. repens*, we argue that there is potential for increased expression in response to a specific cue. Unless it can be shown that an environmental cue leads to altered concentrations of the precursors, it is more practically useful to consider cyanogenesis to be a constitutive system.

It is possible that herbivory in other contexts or by alternate herbivores elicits a different response. Induction of chemical defence often requires a specific pattern of feeding or chemical cue (e.g. Hartley and Lawton, 1991; Raffa, 1991); feeding on *T. repens* by another of its many herbivores could possibly provoke increased cyanogenesis. However, snails are known to be one of the primary agents of clover mortality at the site where the seeds were collected for this study (I.M. Parker and G.S. Gilbert, in prep.). Therefore, feeding by snails should have triggered a response if such a response were an adaptation to local herbivory.

Plants that had experienced drought showed a significant decrease in cyanogenesis in samples to which linamarin had been added, but exhibited no change in samples supplemented with water or enzymes. One explanation is that one or both of the cyanogenic enzymes (linamarase and hydroxynitrile lyase) weakens activity in drought-stressed tissues. Decreased enzyme activity would lower the linamarin concentration at which the enzyme was saturated. If this lowered saturation level were less than the cyanoglucoside concentration in the supplemented samples, but greater than the cyanoglucoside concentration in unsupplemented wells, one would expect to see the pattern we observed: less HCN produced by drought-stressed leaf tissue relative to the control only when linamarin was added.

Such a diminished activity may arise either if the plant's rate of enzyme production is reduced (e.g. by lowered rates of transcription) or if the enzymes' catalytic function is impaired (e.g. by denaturation due to high intracellular salt concentrations). The former would suggest downregulation on the part of the plant, while the latter suggests a physiological limitation. The work reported here does not address whether a decrease in linamarase activity may serve an adaptive function. However, while Foulds and Grime (1972) found that cyanogenic morphs of *T. repens* were subject to drought-induced deaths, it was the production of cyanoglucosides, not linamarase, which led to higher mortality rates. Based on their results, we would have predicted the downregulation of cyanoglucosides rather than enzyme, if chemical plasticity were an adaptive response. We saw the reverse, suggesting that the change in enzyme activity may have been a response to the altered chemiosmotic environment.

In both temperature experiments and in all three tests, cold-treated clones were less strongly cyanogenic than controls. These results are consistent with Till's (1987) observation that populations of *T. repens* at higher elevations show weaker expression than those at lower elevations, although they imply the difference Till observed may have been due to phenotypic modulation rather than genotypic differences among plants. Our findings are also in accord with those of Collinge and Hughes (1982), who showed that cyanoglucoside content in three individuals of *T. repens* was maximized at moderate constant temperatures (around 18°C) and decreased at extremes.

As for the drought experiment, it is unclear whether the reduction in cyanogenesis arises from an actual downregulation on the part of the plant or from a physiological constraint, such as a slowing of the enzymatic pathway to produce cyanoglucosides. Either scenario has the potential to alter the way we think about the balance of fitness costs and benefits that has long been thought to maintain the polymorphism.

The reduction in cyanogenesis in cold temperatures could have an adaptive value. One of the earliest patterns noted in the polymorphism's distribution was a tendency towards higher proportions of acyanogenic morphs in cold climates and high altitudes (Daday, 1954a,b). Daday attributed this pattern to a trade-off between a benefit to cyanogenesis, in the form of herbivore defence, and a cost in decreased cold tolerance due to autotoxicity from cyanide released by damaged cells. The importance of both forces has been supported by more recent research (Dirzo and Harper, 1982a,b; Kakes, 1987; Viette *et al.*, 2000). If a quantitative decrease in cyanogenesis mitigates the cost in cold tolerance, it would follow that reduced cyanogenesis in response to cold temperatures will be adaptive.

This is essentially the reverse of an induced defence; it is widely believed that organisms may reduce allocation costs or autotoxicity of a chemical defence by either initiating or increasing expression when the probability of herbivory is high to maximize benefits (reviewed in Karban and Baldwin, 1997). It is conceivable, then, that the opposite is true; *T. repens* could minimize costs by downregulating cyanogenesis when there is a high probability of frost. The origin and mechanism of this cold response in *T. repens* could be addressed using a functional genomics approach. However, if the cold response provides a selective advantage, it may be adaptive whether it is representative of an actual change in gene expression or due to a physiological constraint. Natural selection is expected to preserve traits that increase fitness regardless of their origin (Karbon and Baldwin, 1997).

Several researchers have questioned whether the temperature-related clines seen by Daday are driven less by fitness costs associated with cold than by decreased herbivore activity in cold climates (Kakes, 1987; Till-Bottraud and Gouyon, 1992). Our conclusions are not changed by this interpretation; the downward shift in expression of cyanogenesis in cold environments could equally be a response to a cue that the probability of herbivory is low. Again, if a quantitative decrease mitigates costs associated with either allocation or autotoxicity, *T. repens* may gain a fitness advantage by shifting resources elsewhere when herbivores are not active.

In conclusion, we found that cyanogenesis of *T. repens* is not an induced defence in the classic sense; intracellular concentrations of the required compounds are not increased in response to herbivory. However, we did find plasticity with regard to environmental factors associated with the fitness costs that are widely presumed to maintain the polymorphism. Future studies should (1) determine whether this plasticity affects fitness and (2) reassess the costs and benefits maintaining the genetic variation in this model system.

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#### REFERENCES

- Anstey, R.L. 1987. Astogeny and phylogeny: evolutionary heterochrony in Paleozoic bryozoans. *Paleobiology*, **13**: 21–43.
- Caradus, J.R. and Forde, M.B. 1996. Characterisation of white clover populations collected from the Caucasus and high altitude regions of eastern Turkey. *Genet. Res. Crop Evol.*, **43**: 143–155.

- Chow, V. 2000. BML's Coastal Conditions. *Bodega Marine Laboratory Homepage*, University of California at Davis, available at <http://www.bml.ucdavis.edu/bmllocal.html>.
- Collinge, D.B. and Hughes, M.A. 1982. Developmental and physiological studies on the cyanogenic glucosides of white clover. *J. Exp. Bot.*, **33**: 154–161.
- Corkill, L. 1940. Cyanogenesis in white clover (*Trifolium repens* L.). I. Cyanogenesis in single plants. *N.Z. J. Sci. Tech.*, **22**: 65b–67b.
- Corkill, L. 1942. Cyanogenesis in white clover (*Trifolium repens* L.). V. The inheritance of cyanogenesis. *N.Z. J. Sci. Tech.*, **23**: 178b–193b.
- Daday, H. 1954a. Gene frequencies in wild populations of *Trifolium repens*. I. Distribution by latitude. *Heredity*, **8**: 61–78.
- Daday, H. 1954b. Gene frequencies in wild populations of *Trifolium repens*. II. Distribution by altitude. *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. Mechanisms of natural selection. *Heredity*, **20**: 355–365.
- Dirzo, R. and Harper, J.L. 1982a. Experimental studies on slug–plant interactions. III. Differences in the acceptability of individual plants of *Trifolium repens* to slugs and snails. *J. Ecol.*, **70**: 101–117.
- Dirzo, R. and Harper, J.L. 1982b. Experimental studies on slug–plant interactions. IV. The performance of cyanogenic and acyanogenic morphs of *Trifolium repens* in the field. *J. Ecol.*, **70**: 119–138.
- Fisher, R.A. 1941. The theoretical consequences of polyploid inheritance for the mid style form in *Lythrum salicaria*. *Ann. Eugen.*, **11**: 31–38.
- Fisher, R.A. 1944. Allowance for double reduction in the calculation of genotype frequencies with polysomic inheritance. *Ann. Eugen.*, **12**: 169–171.
- Foulds, W. and Grime, J.P. 1972. The response of cyanogenic and acyanogenic phenotypes of *Trifolium repens* to soil moisture supply. *Heredity*, **28**: 181–187.
- Green, T.R. and Ryan, C.A. 1972. Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. *Science*, **175**: 776–777.
- Hartley, S.E. and Lawton, J.H. 1991. Biochemical aspects and significance of the rapidly induced accumulation of phenolics in birch foliage. In *Phytochemical Induction by Herbivores* (D.W. Tallamy and M.J. Raupp, eds), pp. 245–276. New York: Wiley.
- Hartvigsen, G. and McNaughton, S.J. 1995. Tradeoff between height and relative growth rate in a dominant grass from the Serengeti ecosystem. *Oecologia*, **102**: 273–276.
- Hughes, M.A. and Dunn, M.A. 1985. The use of immunochemical techniques to study plant proteins. *Plant Mol. Biol. Reporter*, **3**: 17–23.
- Hughes, M.A. and Stirling, J.D. 1982. A study of dominance at the locus controlling cyanoglucoside production in *Trifolium repens* L. *Euphytica*, **31**: 477–483.
- Hughes, M.A., Sharif, A.L., Dunn, M.A. and Oxtoby, E. 1988. The molecular biology of cyanogenesis. In *Cyanide Compounds in Biology*, pp. 111–130. Ciba Foundation Symposium No. 140. Chichester: Wiley.
- Kakes, P. 1985. Linamarase and other  $\beta$ -glucosidases are present in the cell walls of *Trifolium repens* L. leaves. *Planta*, **166**: 156–160.
- Kakes, P. 1987. On the polymorphism for cyanogenesis in natural populations of *Trifolium repens* L. in the Netherlands I. Distribution of the genes Ac and Li. *Acta Bot. Neerland.*, **36**: 59–69.
- Kakes, P. 1991. A rapid and sensitive method to detect cyanogenesis using microtiter plates. *Biochem. Syst. Ecol.*, **19**: 519–522.
- Kakes, P. 1997. Difference between the male and female components of fitness associated with the gene Ac in *Trifolium repens*. *Acta Bot. Neerland.*, **46**: 219–223.
- Karban, R. and Baldwin, I.T. 1997. *Induced Responses to Herbivory*. Chicago, IL: University of Chicago Press.

- Linhart, Y.B. and Thompson, J.D. 1999. Thyme is of the essence: biochemical polymorphism and multi-species deterrence. *Evol. Ecol. Res.*, **1**: 151–171.
- Lively, C.M. 1999. Developmental strategies in spatially variable environments: barnacle shell dimorphism and strategic models of selection. In *The Ecology and Evolution of Inducible Defenses* (R. Tollrian and C.D. Harvell, eds), pp. 245–258. Princeton, NJ: Princeton University Press.
- Melville, J. and Doak, B.W. 1940. Cyanogenesis in white clover (*Trifolium repens* L.). II. Isolation of the glucosidal constituents. *N.Z. J. Sci. Tech.*, **22**: 67b–70b.
- Metcalf, A.L. and Smartt, R.A. 1997. Land snails of New Mexico. In *New Mexico Museum of Natural History and Science: Bulletin 10*. Albuquerque, NM: University of Texas at El Paso and The New Mexico Museum of Natural History and Science.
- Pederson, G.A., Faitbrother, T.E. and Greene, S.L. 1996. Cyanogenesis and climatic relationships in US white clover germplasm collection and core subset. *Crop Sci.*, **36**: 427–433.
- Poulton, J.E. 1990. Cyanogenesis in plants. *Plant Physiol.*, **94**: 401–405.
- Raffa, L.F. 1991. Induced defensive reactions in conifer–bark beetle systems. In *Phytochemical Induction by Herbivores* (D.W. Tallamy and M.J. Raupp, eds), pp. 245–276. New York: Wiley.
- Saucy, F., Studer, J., Aerni, V. and Schneiter, B. 1999. Preference for acyanogenic white clover (*Trifolium repens*) in the vole *Arvicola terrestris*: I. Experiments with two varieties. *J. Chem. Ecol.*, **25**: 1441–1454.
- Schlichting, C. 1986. The evolution of phenotypic plasticity in plants. *Annu. Rev. Ecol. Syst.*, **17**: 667–693.
- Schmitt, J. 1997. Is photomorphogenic shade avoidance adaptive? Perspectives from population biology. *Plant Cell Environ.*, **20**: 826–830.
- Seigler, D.S. 1991. Cyanide and cyanogenic glycosides. In *Herbivores: Their Interactions with Secondary Plant Metabolites*, Vol. I (G.A. Rosenthal and M.R. Berenbaum, eds), pp. 35–77. San Diego, CA: Academic Press.
- Sinervo, B. and Lively, C.M. 1996. The rock–paper–scissors game and the evolution of alternative male strategies. *Nature*, **380**: 240–243.
- Steele, C.L., Lewinsohn, E. and Croteau, R. 1995. Induced oleoresin biosynthesis in grand fir as a defense against bark beetles. *Proc. Natl Acad. Sci. USA*, **92**: 4164–4168.
- Till, I. 1987. Variability of expression of cyanogenesis in white clover (*Trifolium repens* L.). *Heredity*, **59**: 265–271.
- Till-Bottraud, I. and Gouyon, P.H. 1992. Intra- versus interplant Batesian mimicry? A model on cyanogenesis and herbivory in clonal plants. *Am. Nat.*, **139**: 509–520.
- Viette, M., Tettamanti, C. and Saucy, F. 2000. Preference for acyanogenic white clover (*Trifolium repens*) in the vole *Arvicola terrestris*: II. Generalization and further investigations. *J. Chem. Ecol.*, **26**: 101–122.
- Wright, S. 1931. Evolution in Mendelian populations. *Genetics*, **16**: 97–159.