Journal of Plant Biology, June 2004, 47(2) : 124-128

Influence of Two Selective Factors on Cyanogenesis Polymorphism of *Trifolium repens* L. in Darjeeling Himalaya

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Cyanogenesis-the production of toxic hydrogen cyanide (HCN) by damaged tissue-in *Trifolium repens* L. (white clover), a type of most important pasture legume, has been studied at different elevations of Darjeeling Himalaya (latitude-27° 2' 57" N, longitude- 88° 15' 45" E). Release of HCN takes place due to reaction between cyanogenic glucosides stored in vacuoles of the leaf cell and the corresponding enzyme β-glucosidase present in another compartment, often cell wall. Cyanogenesis, a defense system in plant, protects the clover from herbivore and inhibits grazing. Biochemical analysis showed the presence and absence of the cyanogenesis trait within the population in different proportions at different elevations. Acyanogenic individuals also showed variations with respect to presence or absence of either cyanogenic glucosides or β-glucosidase enzyme or both. The distribution of cyanogenic and acyanogenic plants was found in all places, but at lower altitudes (2084-2094 m) the dominating plants were cyanogenic whereas in higher altitude (2560 m) the dominating plants were acyanogenic. It was observed that blister beetle (*Mylabris pustalata* Thunb.) and the mollusc (*Macrochlamys tusgurium* Benson.) were the most common consumer of leaflets of *T. repens*. Six categories of damage on white clover leaf by these animals were recorded. Our results suggest that the two selective factors or forces i.e. very cold temperature (harmful to cyanogenic plants) at higher altitude as well as indiscriminate but preferential predation (harmful to acyanogenic plants) interact to affect the system of cyanogenesis and also to cause the stable and protective polymorphism in *T. repens* rather than genotypic differences present among the plants.

Keywords: cyanogenesis, polymorphism, selective factors, *Trifolium repens* L.

The genetic polymorphism of white clover (*Trifolium repens* L.) has long been a model system for the study of genetic variability. A second process that can lead to polymorphism is the cyanogenesis. It may even act in an explicitly manner to allow the plant to express the genotype best suited for a particular environment. Cyanogenesis is the production of volatile-poisonous hydrogen cyanide (HCN) by the damaged tissue of plant. The ability to release hydrogen cyanide (HCN) after tissue damage requires the presence within the plant tissue of both cyanoglucoside (in white clover, a mixture of lotaustralin and linamarin: Melville and Dock, 1940) and β-glucoside enzyme, linamerase (Corkill, 1940). A single dominant gene, Li, controls inheritance of linamerase, while production of both linamarin and lotaustralin is controlled by the dominant gene, Ac (Corkill, 1942). The production of cyanide follows two steps. In the first step, the sugar is cleaved from cyanoglucoside by glycosidase. The resulting cyanohydrin is then hydrolyzed by a hydroxynitrile lyase, releasing hydrogen cyanide (Poulton, 1990). Cyanoglucosides are not broken down in the intact plant, because the glucosides are stored in the cell vacuoles and the degradative enzymes are present in the cell wall. Under normal condition, this compartmentalization prevents decomposition of the glucoside. However, when the tissue is crushed or damaged, as during herbivore feeding, the cell contents of different tissue mix and HCN is formed (Kakes, 1985; Crush and Caradus, 1995). The leaf of white clover produces hydrogen cyanide when herbivores like insects, snails and slugs damage it. Therefore, it develops into a type of chemical defense mechanism against the minor herbivores. Release of poisonous HCN makes the unpleasant taste of the leaf and perhaps by this way the plant can protect it form herbivore. To date, most studies of cyanogenesis of white clover have focused on the presence or absence of the trait, although there is evidence of variability in expression within individuals (Hayden and Parker, 2002). The present work here extends the investigation on biochemical analysis and survey of cyanogenic and acyanogenic populations at different elevations of Darjeeling Himalaya and to detect any selective factor that might interact with cyanogenesis in *T. repens*.

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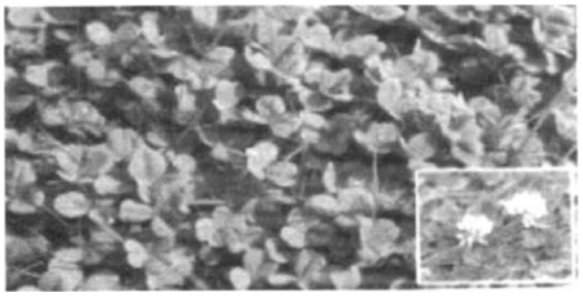

Figure 1. Vegetation of *T. repens* L., and inflorescence within inset.

MATERIALS AND METHODS

Cyanogenesis in *T. repens* (Fig. 1) was detected by sodium picrate tests. The three terminal leaflets of a stolon, including the youngest (unfolded) leaf about 0.2 g introduced in test tubes (7 cm × 1.7 cm) and two drops of analar toluene were added; the material was then crushed with a glass rod. The leaf materials were collected from Happy valley (altitude, 2084 m), Lloyd Botanical Garden (altitude, 2094 m), Shrubbery Park (altitude, 2134 m), Ghoom (altitude, 2338 m) and Jalapahar (altitude, 2560 m). Strips of sodium picrate paper (9.5 cm × 1.3 cm) were suspended in the corked test tubes and incubated at 37°C for 24 h. Plants were scored as cyanogenic after the incubation period; the sodium picrate paper had changed from its yellow-lemon color to some shades of brown. The intensity of the color depends on the concentration of HCN production. Samples that produced no color, were further tested to consider them as acyanogenic phenotypes. In one test tube, two drops of a solution of the cyanogenic glucoside and to the other, two drops of the enzyme were added. The cyanogenic glucoside and the enzymes used for the test were isolated from the leaflets of the cyanogenic plants in our laboratory. One hundred fair sized leaflets into 25 ml of distilled water were autoclaved at 110°C (10 lb pressure) for 15 min. The brown filtered extract of leaflets was used as cyanogenic glucoside source. The enzyme was extracted from the leaflets in phosphate buffer (pH 8.2) at 4°C and centrifuged at 10000g. The leaflets that gave the negative results in three types of test were scored as acyanogenic phenotype.

RESULTS

Cyanogenesis Test in Plant

On the basis of color change or unchange of the picrate paper (Fig. 2a-d), the populations of *T. repens* were broadly classified into two groups: (a) cyanogenic and (b) acyanogenic. Among cyanogenic plant populations, the intensity of the color change of picrate paper showed some variations ranging from deep brown to light brown (Fig. 2a-c). The depth of color depends on the concentration of HCN produced by the crushed tissue. Acyanogenic populations were again grouped into three sub-categories such as:

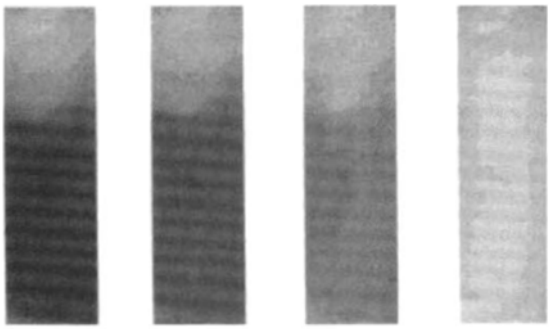

Figure 2. Test of hydrogen cyanide (HCN) on the strips of lemon yellow colored sodium picrate paper. Change to deep brown color (a), medium brown color (b), light brown color (c), and unchanged lemon-yellow color (d) that are shown in darker or lighter images in black and white pictures are apparent.

(i) The first sub-category of acyanogenic plants which produced HCN and showed color change of picrate paper under second test after the addition of extracted glucosides, were classified as positive for the presence of enzyme. It means that in the plant tissue β-glucosidase is present, but the cyanogenic glucoside is absent. Hence, HCN is not produced in natural condition. The production of HCN through the hydrolysis of glucoside was started when glucoside was added in the test system.

(ii) The second sub-category of acyanogenic plant showed the positive test result for cyanogenesis after the addition of extracted enzyme. It means that the enzyme is absent, but cyanogenic glucoside is present.

(iii) The third sub-category of acyanogenic plant showed the color change of picrate paper when both enzyme and glucoside were added at a time. It means that the plant contains neither the enzyme nor the glucoside in natural condition. Therefore, four cyanotypes of *T. repens* can readily be distinguished by simple biochemical tests. The cyanogenic polymorphism in *T. repens* is summarized in Table 1.

Distribution of the Cyanogenesis Polymorphic Plants

In the present investigation, the distribution of cyan-

Table 1. The cyanotype of the cyanogenesis polymorphism in *T. repens*.

Cyanotype	Plant contents	Reaction
C	Cyanogenic glucosides and enzyme	Cyanogenic
AC-1	Cyanogenic glucosides but no enzyme	Acyanogenic
AC-2	Enzyme but no cyanogenic glucosides	Acyanogenic
AC-3	Neither cyanogenic glucosides nor enzyme	Acyanogenic

Table 2. The cyanotype of the cyanogenesis polymorphism in *T. repens*.

Locality	Number and percentage of cyanotype				Total
	C	AC-1	AC-2	AC-3	
Happy Valley (2084 m)	912 (69.30%)	162 (12.31%)	174 (13.22%)	68 (5.17%)	1316
Botanical Garden (2094 m)	930 (70.50%)	157 (11.90%)	170 (12.89%)	62 (4.70%)	1319
Shrubbery Park (2134 m)	543 (42.06%)	379 (29.36%)	241 (18.67%)	128 (9.91%)	1291
Ghoom (2338 m)	212 (18.88%)	415 (36. 95%)	327 (29.19%)	169 (15.05%)	1123
Jalapahar (2560 m)	98 (7.89%)	556 (44.77%)	402 (32.37%)	186 (14.97%)	1242

C, cyanogenic (both cyanogenic glucosides and enzyme present); AC-1, acyanogenic type 1 (enzyme absent); AC-2, acyanogenic type 2 (cyanogenic glucosides absent); AC-3, acyanogenic type 3 (both cyanogenic glucosides and enzyme absent).

ogenic and acyanogenic plants was studied from five different places located at different altitudes. Both cyanogenic and acyanogenic plants were found in all places, but at lower altitude the dominating population was cyanogenic whereas in higher altitude the dominating population was acyanogenic. The detailed analysis of the distribution of cyanogenic and acyanogenic plants is given in Table 2. In order to quantify the cyanotypes, average count of 12 (50 cm^2) quadrates of each locality was taken into consideration.

Effect of the Animals on the Plant

During the period of investigation from August to October, observation was also made to determine which herbivores were potentially important to the plant. It was observed that among several feeders of common garden plants, blister beetle (*Mylabris pustalata* Thunb.) and the mollusc (*Macrochlamys tusgurium* Benson.) was the most common consumer of leaflets of *T. repens*. By these animals, six categories of damage on white clover leaf (Fig. 3) were recorded: untouched (U), nibbled (N), up to 25% of the leaflet taken (A), 25-50% taken (B), 50-75% taken (C) and 75-100% of the leaflet removed (D). From such observations, it was possible to establish a system of scoring animal damage based on the area removed from leaflets of two broad cyanotypes. In order to identify accurately and quantify the damage, average count of 12 (50 cm^2) quadrates of each locality was taken. The detailed analysis of the result is given in Table 3.

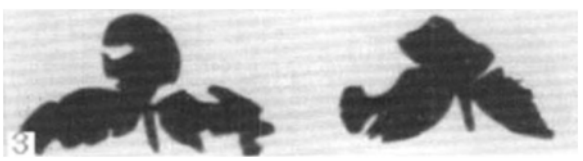

Figure 3. Damaged leaflets by insect and mollusc.

DISCUSSION

We did not find increased cyanogenesis in response to feeding by insect and mollusc herbivore. In the light of these finding it would appear that cyanogenesis of *T. repens* is not inferred in the strict sense by herbivore. This system is not induced defense in the classical sense rather it is a constitutive system. The defensive role of cyanogenesis is only expressed when the enzymatic hydrolysis of the glucoside by β-glucosidase takes place in the damaged tissue. In absence of such chemical reaction due to either lack of enzyme or glucosides or both, makes the plant acyanogenic (Table 1), undefended and polymorphic. Since the cyanogenesis polymorphism in *T. repens* does not appear to be associated with any aposematic features (i.e. the plants are not conspicuously colored or marked) and the animals can not discriminate the polymorphism, therefore random grazing may be the only mechanism by which animals can determine the acceptability or unacceptability of potential food. Table 3 shows the contingency analyses of the result of random grazing by *M. pustalata* Thunb. and *M. tusgurium* Benson.

In the present investigation, the occurrence and coexistence of cyanogenic and acyanogenic cyanotypes

have been observed in different proportions in the same habitat and it has some adaptive value in nature. The advantage of co-existence is that it possibly deludes the predators and gives the protection to the acyanogenic cyanotypes (AC-1, AC-2, and AC-3) from herbivores to some extent. However, β-glucosidase is also reported to occur in the digestive tract of animals, for example, in insects (Bernays, 1977) and mollusc (Runham and Hunter, 1970). Their enzyme is capable of hydrolyzing the cyanogenic glucosides present in plant. Therefore, it seems likely that molluscs and insects that feed on acyanogenic plant (Saucy et al., 1999; Viette et al., 2000) containing cyanogenic glucosides but not enzyme, will experience the cyanogenic reaction in their digestive tract though not at the time of tasting. Therefore, the co-existence of acyanogenic cyanotypes having no glucosiodes (AC-2, AC-3) with AC-1 cyanotype (having glucosides) possibly gets some protection from herbivore.

It has been observed (Table 2) that the number of acyanogenic cyanotypes is more frequent towards higher altitude i.e. towards the colder regions (3-7°C) such as Ghoom, Jalapahar, but the number of cyanogenic (16-20°C) such as Happy Valley, Botanical Garden is not. It appears that very cold temperatures are capable of activating the enzyme while the plant is still intact (Pusey, 1965) and causes decreased cold tolerance of cyanogenic population due to auto toxicity from cyanide. The consequent internal release of HCN actually inhibits the cellular respiration and puts the cyanogenic plant exposed to such cold conditions at an evident disadvantage presumably outweighing the advantage they would obtain by deterring their predators. Kakes (1987) found that cyanomorph frequency did not correspond to altitude or temperature

Table 3. Number of cyanogenic and acyanogenic leaflets damaged by *M. pustalata* Thunb. and *M. tusgurium* Benson. Analyses (χ^2) are shown for individual locality.

a) *M. pustalata* Thunb.

Locality	Cyanotype	Category of damage				Total	χ^2	d.f.	P
		U	N	A+B	C+D				
Happy Valley (2084 m)	Cyanogenic	50	3	10	1	64	16.2	3	<0.005
	Acyanogenic	22	2	10	10	44			
Botanical Garden (2094 m)	Cyanogenic	46	2	11	12	71	1.0	3	>0.75
	Acyanogenic	31	1	10	12	54			
Shrubbery Park (2134 m)	Cyanogenic	46	6	13	1	66	35.6	3	<0.001
	Acyanogenic	20	1	11	21	53			
Ghoom (2338 m)	Cyanogenic	35	9	7	1	52	22.1	3	<0.001
	Acyanogenic	21	3	19	11	54			
Jalapahar (2560 m)	Cyanogenic	29	4	8	6	47	12.1	3	<0.01
	Acyanogenic	24	1	10	23	58			

b) *M. tusgurium* Benson.

Locality	Cyanotype	Category of damage				Total	χ^2	d.f.	p
		U	N	A+B	C+D				
Happy Valley (2084 m)	Cyanogenic	74	1	2	23	100	14.5	3	<0.005
	Acyanogenic	24	2	5	23	54			
Botanical Garden (2094 m)	Cyanogenic	70	2	4	3	79	48.8	3	<0.001
	Acyanogenic	45	1	3	45	94			
Shrubbery Park (2134 m)	Cyanogenic	65	3	9	6	83	29.1	3	<0.001
	Acyanogenic	39	1	1	28	69			
Ghoom (2338 m)	Cyanogenic	63	4	9	13	89	42.9	3	<0.001
	Acyanogenic	36	1	2	56	95			
Jalapahar (2560 m)	Cyanogenic	60	2	1	10	73	11.2	3	<0.01
	Acyanogenic	21	1	3	14	39			

U, untouched; N, nibbled; A+B, 25-50% of leaflet damaged; C+D, 70-100% of leaflet damaged.

cline. Till (1987) found that white clover at higher altitude was more like to be only weakly cyanogenic. Pederson et al. (1996) found that the temperature and altitude correlation was upheld in the United States, and Caradus and Forde (1996) confirmed the patterns in Western Turkey. Furthermore, Hayden and Parker (2002) reported that some genotypes of *T. repens* showed significantly weaker expression of cyanogenesis in very cold temperature could have an adaptive value and could equally be a response to a cue that the possibility of herbivore is low. Therefore, on the basis of distribution pattern of cyanogenic and acyanogenic white clover at different altitudes and temperature clines, it can be concluded that the two opposing selective factors or forces i.e. very cold temperature (harmful to cyanogenic plants) at higher altitude as well as indiscriminate but preferential predation (harmful to acyanogenic plants) influence the system of cyanogenesis and also causes the protective polymorphism in *T. repens* rather than genotypic differences present among the plants as observed in the present investigation.

Received November 6, 2004; accepted March 16, 2004.

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