Theor Appl Genet (1989) 77:111–118

An analysis of the costs and benefits of the cyanogenic system in *Trifolium repens* L.

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Received June 15, 1988; Accepted June 22, 1988
Communicated by P.M.A. Tigerstedt

Summary. The effect of the cyanogenic glucosides linamarin and lotaustralin and their hydrolyzing enzyme linamarase was studied in a B_2 generation segregating for the genes *Ac* and *Li*. Plants containing the glucosides are protected against grazing by snails both in the seedling stage and as adult plants. In seedlings, however, there is a direct effect on survival, whereas in adult plants the leaf area of plants containing linamarin/lotaustralin is less reduced under intense grazing. Linamarase has no effect on grazing by snails, possibly as a result of the presence of β-glucosidase activity in the gut of these animals. The genes *Ac* and *Li*, or genes tightly linked to them, have other effects as well: plants possessing one dominant *Ac* allele produce fewer flowers than homozygous *ac* plants. I compared this difference in flower production to the metabolic cost of producing the cyanogenic glucosides. The energy content of the difference in flower head production far exceeded the metabolic cost of cyanoglucoside production in *Acac* plants. It is possible that the cost of maintaining a certain level of cyanoglucosides is much more important for the plant than the initial cost of biosynthesis. The importance of the effects of *Ac* and *Li* in the maintenance of cyanogenic polymorphism in white clover is discussed.

Key words: Cyanogenesis – Linamarin – Linamarase – *Trifolium repens*

Introduction

The polymorphism for cyanogenesis has been studied ever since its discovery by Ware (1925). Two genes, *Ac* and *Li*, are responsible for this polymorphism: the first regulates the presence of the cyanogenic glucosides linamarin and lotaustralian; the second, the presence of the β-glucosidase linamarase. Both *Ac* and *Li* show incomplete dominance (Hughes and Stirling 1982; Hughes et al. 1984; Maher and Hughes 1973), but as the semiquantitative tests used in most of the ecological and genetical studies do not distinguish between homozygote dominant plants and heterozygote ones, the dominance is generally considered to be complete.

Although the four phenotypes can be distinguished relatively easily with semiquantitative tests, at least in some genetic backgrounds, the majority of ecological studies have been performed on cyanogenic (*Ac-, Li-*) versus acyanogenic (*acacLi-, Ac-lili, acaclili*) phenotypes. This situation is rather unfortunate because the effects of *Ac* and *Li* not only on cyanogenesis but also on growth, reproduction and protection against grazing can be very diverse. Moreover, the study of two genes showing interaction and polymorphism in natural populations is interesting both from the theoretical and from the practical point of view.

The studies published so far in which *Ac* and *Li* were distinguished report a number of differences associated with the two genes. A compilation of the results is given in Table 1. In comparing these data, serious difficulties arise: the plants used by the different authors have very different genetic backgrounds. Most have been derived from commercial varieties; some have been taken from natural populations in different parts of the world. In only one study were young plants (seedlings) used; the others used (cuttings of) adult plants. The conditions under which the plants were grown were also variable: controlled climate room, greenhouse, open field. An analysis of the costs and benefits should take into account the whole life cycle of the plant. Moreover, the environmental conditions under which the plants are studied should not depart too much from the natural

Table 1. Comparison of the secondary effects of *Ac* and *Li* as reported in the literature

Character involved	Gene(s) involved	Effect	Reference	Confirmed in this study
No. of flowers	*Ac*	*Ac* < *ac* in cool conditions	Daday 1965	Yes
No. of flowers		*Ac* > *ac* in warm conditions	Daday 1965	–
No. of flowers	*Ac* and *Li*	*Ac, Li* < *Acli, acli, acli*	Foulds and Grime 1972	Partly
No. of flowers	*Ac* and *Li*	*Ac, Li* < *Acli, acli, acli*	Dirzo and Harper 1982b	–
Vegetative yield	*Ac* and *Li*	*AcLi* > *Acli, acLi, acli* in warm conditions	Daday 1965	–
Drought resistance	*Ac*	*Ac* > *ac*	Foulds and Grime 1972	–
Leaf area	*Li*	*Li* > *li*	Ennos 1981a	No
Competitive ability	*Li*	Highest in mixed stands	Ennos 1981b	–
Early establishment	*Li* (+*Ac*?)	*Li* > *li*	Ennos 1981b	–
Root growth	*Li*	*Li* > *li*	Dommée et al. 1980	–
Protection to grazing of adult plants	*Ac*+*Li*	*AcLi* > *Acli, acli, acli*	Dirzo and Harper 1982a	only *Ac*

conditions of the populations polymorphic for the *Ac* and *Li* genes.

It is very unlikely that the polymorphism for cyanogenesis is neutral. The latitudinal and altitudinal clines found by Daday (1954a, b) and confirmed by others (Aranjo 1976; Till-Bottraud et al. 1988) and the presence of linkage disequilibrium (Ennos 1982; Kakes 1987) argue against this. A problem inherent to the study of natural selection is how to separate the effects of the genes under study from the effects of the genetic background and from genes linked to and/or in linkage disequilibrium with the former. Several schemes have been proposed to minimize the effects of other genes. The method used most often is to compare plants with different phenotypes from one population. When the sample is large enough, we may assume that the genetic background is randomized. Only large populations with approximately equal percentages of the phenotypes can be used in this approach. The effects of linked genes and other genes in linkage disequilibrium with the ones under study are not avoided in this way. As we know that *Ac* and *Li*, being unlinked, show a marked linkage disequilibrium in British (Ennos 1982) and Dutch (Kakes 1987) populations, the interaction of *Ac* and *Li* (and of genes closely linked to them) should be taken into account in a study of the effects of cyanogenic loci.

I have used a backcross segregating for *Ac* and *Li* to study the effects of these genes. *Trifolium repens* is an obligate outbreeder with a high level of intrapopulation differentiation (Burdon 1980). Any one plant taken from a natural population will be highly heterozygotic, and consequently the plants in successive backcross generations will be far from uniform. The backcross used for this study segregated for two morphological markers and for four out of the eight isoenzyme markers studied (none of these markers were linked to *Ac*/*Li*). The disequilibrium of genes not physically linked will be absent, but the effects of genes linked to *Ac* or *Li* may still be present after many generations of backcrossing, provided there is tight linkage. Consequently, when *"Ac"* or *"Li"* are referred to, what is actually meant is a chromosome segment of unknown length around these genes. This is implicit for all research on *Ac*/*Li* published so far. In this paper, I present an account of the effects of *Ac* and *Li* as defined above at different stages of the life cycle of a B_2 generation.

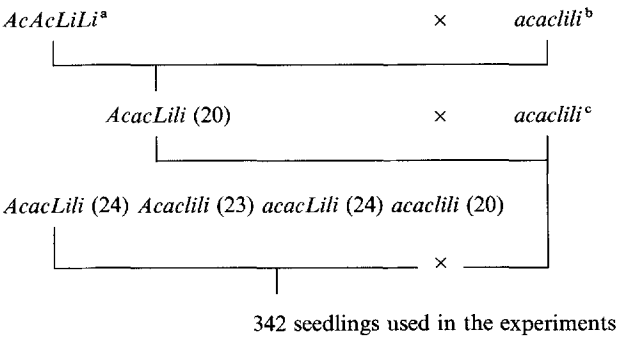
Materials and methods

The parent plants used, TR4506 (*AcAcLiLi*) and TR3803 (*acaclili*), were taken from two populations in the Cevennes (France). Details of these populations are to be found in Boersma et al. (1983). Table 2 gives the crossing scheme used.

In each generation, three plants heterozygous for *Ac* and *Li* were backcrossed. As the resulting families all showed the expected 1 : 1 segregation and did not differ in general phenotype, only one B_2 family was used for the following experiments. Two grazing experiments were conducted in the greenhouse, one on seedlings and one on adult plants. All yield determinations were made on plants growing in the experimental garden. The B_2 consisted of 342 seedlings (germination: 85%); 178 of these were distributed over three trays and grazed upon by snails: four *Helix aspersa* and one *Cepaea nemoralis* per tray. The total grazing period was 18 days, and the snails were rotated over trays every 3rd day. The seedlings were from 5 to 46 days old at the start of the experiment. After a 2-week recovery period, all survivors and the controls (164 plants) were potted, and their cyanotype was determined with the Feigl-Anger test (Kakes 1987).

Grazing on adult plants: The leaf area of 20 plants, 5 of each cyanotype, was determined by measuring the length of the end leaflet of each leaf, using the relation: $Y = 4.5049 \cdot X^{1.7432}$

Table 2. Ancestry of the B_2 used in the experiments



[a] One plant, taken from a natural population: Gaseiral (S. France)
[b] One plant, taken from a natural population: Source de Galadet (S. France)
[c] One plant, taken from a natural population: Col du Pas (S. France)
(n) = Number of plants raised in the greenhouse

where Y is the leaf area in mm^2 and X, the length of the end leaflet in mm. Three plants of each cyanotype were grazed by eight *Helix aspersa* for 7 days. The other plants were kept as controls. After 7 days, the area of all leaves was measured with an area meter.

Field experiment: 10 plants of each of the four cyanotypes were taken from the control plants of the seedling experiment. Two cuttings of each plant were rooted in the greenhouse. The 80 plants were transferred to the experimental garden on the grounds of the Free University in July 1985 in a completely randomized plot with a plant distance of 60 cm. From August to November 1985, the flower heads were collected after the stalks had withered. In November 1985 all remaining flower heads were collected, and the lengths of the three longest stolons on each plant were determined.

The measurements were repeated in June 1986. After June 23rd 1986, the ripe flower heads were collected twice a week. Between July 23rd and August 1st, the plants were harvested by digging them up and removing the soil from the roots as much as possible. The plants were separated into ripe flower heads, unripe flower heads (stalks not withered) and vegetative parts. All plant material was oven dried at 70 °C for 24 h. Subsamples of the collected material were finely ground and used for the following determinations: (1) elementary composition – C, H and N – was determined with a Carlo Erba elemental analyzer model 1106; (2) other mineral nutrients were determined by atomic absorption spectrometry after digestion with strong acid ($HNO_2 : HClO_4$, 7 : 1, v/v); (3) caloric values were calculated with the microbomb method of Phillipson (1964).

Results

Grazing experiment on seedlings

Table 3 shows the cyanotypes of the survivors of the grazing experiment and of the control plants. It is clear from this table that plants possessing linamarine/lotaustraline have a much better chance of surviving than plants lacking the cyanogenic glucosides: of the survivors of the grazing experiment, plants possessing the cyanogenic glucosides outnumber those lacking it by 5 : 1, whereas the control plants show the expected 1 : 1 ratio. The presence of linamarase had no significant effect on survival and there was also no significant interaction of *Ac* and *Li* with regard to survival. All plants had been grazed to some extend at the end of the grazing period.

Table 3. Results of the seedling experiment

A: Grazed seedlings (survivors of 178 plants)

	Lili	*lili*	
Acac	30	20	50
acac	5	5	10
	35	25	60
χ^2 tests:	χ^2	*df*	*P*
Ac-ac (1:1)	26.667	1	< 0.001
Li-li (1:1)	1.667	1	0.20
Interaction *Ac-Li-*	0.343	1	0.56

B: Ungrazed seedlings (164 plants at the start of the experiment)

	Lili	*lili*	
Acac	36	42	78
acac	52	34	86
	88	76	164
χ^2 tests:	χ^2	*df*	*P*
Ac-ac (1:1)	0.390	1	0.53
Li-li (1:1)	0.878	1	0.35
Interaction *Ac-Li*	3.369	1	0.07

Table 4. Analysis of variance of the grazing index of the four cyanotypes

Source	SS	DF	MS	F-Ratio	Prob > F
Between *Ac*	9,404.360	1	9,404.360	11.043	0.010
Between *Li*	1,007.179	1	1,007.179	1.183	0.207
Interaction	10.240	1	10.240	0.012	0.915
Error	6,813.178	8	851.647		
Total	17,234.957	11			

Grazing experiment on adult plants

To study the effect of grazing by snails on adult plants, 20 plants were used in a greenhouse experiment as described in the section the Material and methods. The difference in leaf area (ΔLA) is the result of growth (controls) and growth versus grazing (grazed plants). The control plants had ΔLA values ranging from 52 to 134 cm^2. As there was no significant effect of *Ac* and/or *Li* on LA and ΔLA of the control plants, the mean ΔLA of the controls (92 cm^2) was used to construct a grazing index: ΔLA of grazed cyanotype/ΔLA of controls multiplied by 100. The results are shown in Fig. 1 and Table 4.

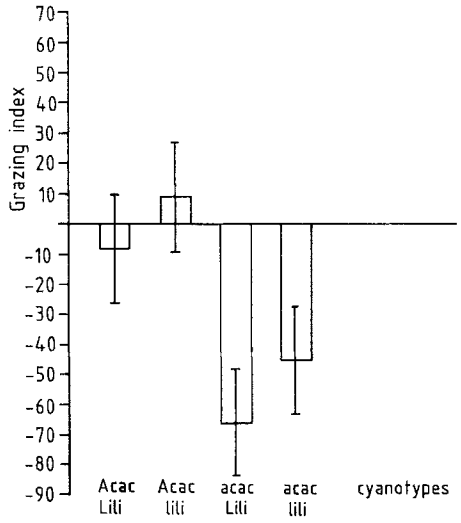


Fig. 1. Grazing index of the cyanotypes of *T. repens*

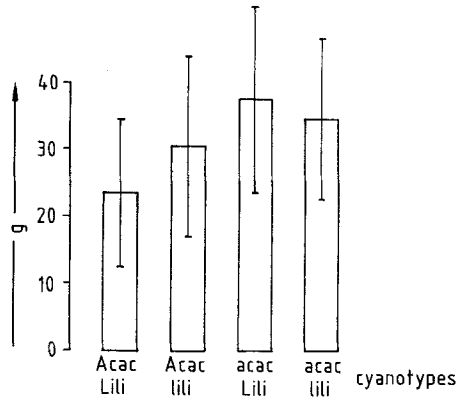


Fig. 2. Mean dry weight of the vegetative parts of the cyanotypes of *T. repens*

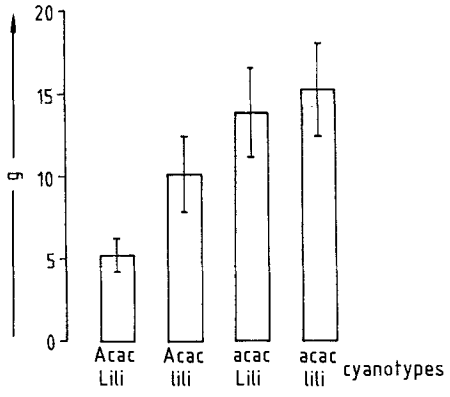


Fig. 3. Mean dry weight of the ripe flower heads of the cyanotypes of *T. repens*

Table 5. Analysis of variance of the vegetative yield of the four cyanotypes

Source	SS	DF	MS	F-Ratio	Prob > F
Between *Ac*	1,405.158	1	1,405.158	1.898	0.116
Between *Li*	36.775	1	36.775	0.050	0.824
Interaction	1,217.112	1	1,217.112	1.644	0.119
Error	56,272.129	76	740.423		
Total	58,931.174	79			

Table 6. Analysis of variance of the reproductive yield of the four cyanotypes

Source	SS	DF	MS	F-Ratio	Prob > F
Between *Ac*	945.038	1	945.038	8.924	0.004
Between *Li*	196.627	1	196.627	1.857	0.117
Interaction	63.084	1	63.084	0.596	0.443
Error	8,048.134	76	105.896		
Total	9,252.882	79			

The effect of *Ac* is significant: *acac* plants have lower grazing indices than *Acac*, *Li* has no effect and there is also no *Ac*/*Li* interaction.

Field experiment

Vegetative yield. Figure 2 shows the mean and l.s.d. (95%) of the dry weight of the vegetative parts and Table 5 gives the ANOVA table. There is no significant effect of *Ac* and/or *Li* on vegetative yield.

Reproductive yield

The dry weight of the ripe flower heads collected between June 23rd and August 1st of 1986 was used as a measure for the reproductive yield of the plants. Figure 3 and Table 6 give the results of the dry weight of ripe flowers and the analysis of variance. *Ac* has a significant effect on yield: plants without linamarin/lotaustralin produce twice as much reproductive dry weight as plants with cyanogenic glycosides. The effect of *Li* is not significant, and there is also no significant *Ac*/*Li* interaction.

In order to assess the relative contribution of the *Ac* gene, unidentified genes influencing yield and the environment on the variation observed, we performed a nested ANOVA on the yield data. Table 7 shows that the cyanotype effect (comprising *Ac* and *Li*) is significant, whereas the within cyanotypes effect that measures genetic differences not linked to *Ac* or *Li* is not significant.

Total yield. The total yield (vegetative parts plus all flower heads collected) showed the same trend as that for

Table 7. Nested analysis of variance of the reproductive yield of the four cyanotypes

Source	SS	DF	MS	F-ratio	Prob > F
Cyanotype	1,281.78	3	427.26	3.39	0.028
Within cyanotypes	4,536.76	36	126.02	1.48	0.110
Within clones	3,407.34	40	85.18		
Total	9,225.88	79			

Note: The slight difference in total SS between tables 6 and 7 is due to a difference in the handling of missing values

Table 8. Mean and standard deviation of the caloric value of the flower heads and vegetative parts of *Trifolium repens*

Part	Number of determinations	Mean	± 1 S.D.
Flowerheads	10	18.8 kJ/g	0.7 kJ/g
Vegetative parts	8	14.3 kJ/g	2.9 kJ/g

Table 9. Elementary composition of the flowers and vegetative parts of *Trifolium repens*

Part	Cyano-type(s)	Ele-ment	Unit	Quantity ± standard error
Flower	All	N	%	2.54 ± 0.15
		C	%	39.11 ± 1.11
		H	%	5.58 ± 0.17
		K	mmol kg^{-1}	37.5 ± 3.22
		Na	mmol kg^{-1}	1.69 ± 0.29
		Ca	mmol kg^{-1}	35.4 ± 2.65
		Mg	mmol kg^{-1}	6.72 ± 0.32
		Fe	mmol kg^{-1}	1.71 ± 0.36
		Mn	µmol kg^{-1}	71.7 ± 9.21
		P	mmol kg^{-1}	6.80 ± 0.60
Vegetative	All	N	%	2.03 ± 0.04
		C	%	37.0 ± 0.82
		H	%	5.58 ± 0.13
		K	mmol kg^{-1}	25.9 ± 1.69
		Na	mmol kg^{-1}	2.39 ± 0.28
		Ca	mmol kg^{-1}	56.10 ± 6.23
		Mg	mmol kg^{-1}	6.69 ± 0.40
		Fe	mmol kg^{-1}	2.85 ± 0.39
		Mn	µmol kg^{-1}	224 ± 124
		P	µmol kg^{-1}	5.60 ± 0.39
All	*Acac*	Mg	mmol g^{-1}	6.07 ± 0.20
	acac	Mg	mmol g^{-1}	7.33 ± 0.33

the reproductive yield, but the differences between cyanotypes were not significant owing to the large within genotypes component of the variation.

Caloric value and elementary composition

In order to evaluate the difference in reproductive yield, a comparison was made of the mineral content and the caloric value of the different parts of the four cyanotypes. A subset of four plants was used to measure the caloric value of the flower heads and vegetative parts and a subset of eight plants to study the mineral composition. There was no significant difference between the caloric values of cyanotypes, both for flower heads and vegetative parts. The means and standard deviations are given in Table 8.

The elementary composition of the flower heads and vegetative parts is given in Table 9. There is no effect of cyanotype of the C, H and N composition. Of the minerals, only Mg is influenced by the cyanotype: *Acac* plants have a lower Mg content than *acac* plants. As the difference in Mg content could be caused by a difference in chlorophyll content, the latter was measured in leaves of the genotypes used in the field studies kept in the greenhouse. No difference between plants could be demonstrated.

Discussion

A comparison of the present results with those of other researchers (Table 1) leads to the following conclusion: the effect of linamarin/lotaustralin on the survival of grazed seedlings has not been published before. Recently, Horrill and Richards (1986) reported a difference in survival between cyanogenic and acyanogenic seedlings of *T. repens* grazed by the slug *Arion hortensis*. They did not, however, study the effect of cyanoglucosides and linamarase separately. Furthermore, their results apply to differences between commercial varieties of white clover, leaving open the possibility that differences in palatability and/or ability to recover not related to *Ac* and/or *Li* influenced the results. It is interesting that their conclusion that the difference is in rate of grazing rather than in absence of damage is corroborated by the present results. We must bear in mind, however, that the linamarin/lotaustralin content of the seedlings in the present study was low: I found 2.8–5.6 µg HCN g^{-1} fresh weight, whereas Horrill and Richards (1986) found 180–246 µg HCN g^{-1} fresh weight.

The effect of *Ac* on flowering was found by Daday (1965) in experiments conducted under cool conditions. Foulds and Grime (1972) and Dirzo and Harper (1982b) found a difference in flower production between *Ac-Li-* and the other three cyanotypes. As shown in Fig. 1, the *AcacLili* genotype actually had the lowest flower production in my study. However, the statistical analysis (Table 6) showed that only the *Ac* effect is significant. The difference in leaflet length and width associated with *Li* reported by Ennos (1981a) was not found in this study. As Ennos used cyanotypes from a natural population, it is possible that the difference in results is caused by gene(s) in linkage disequilibrium with *Li*.

Are the differences found in this and other studies the result of different concentrations of cyanoglucosides or/and hydrolyzing enzymes, or must they be regarded as pleiotropic effects of the *Ac* and *Li* genes or even as effects of polymorphic genes linked to *Ac* and/or *Li*? It is very probable that the effect of the cyanoglucosides on grazing is in fact the result of a causal relation. It is not surprising that linamarase has no effect: Dirzo and Harper (1982a) have shown that the gut content of *Agriolimax reticulatus* has linamarase activity: whereas Kakes (unpublished results) has found that an extract of the gut of *Helix pomatia* hydrolizes linamarine ten times faster than extracts of *LiLi Trifolium* leaves on a dry weight basis. The other effects of *Ac* and/or *Li* found in this study are more difficult to relate directly to the gene products.

The effect of *Ac* on flower production could be the result of a different allocation of energy and/or biomass in plants producing cyanoglucosides and in this way constitute (part of) the cost of chemical defense. The question arises: is the difference in reproductive yield of the same order of magnitude as we would expect from the amount of cyanoglucosides present in the plants? There are two possible answers to this question, depending on what is the limiting factor in cyanoglucoside production – energy or nitrogen. As the plants I used in the experimental field all had extensive nodulations with the symbiontic nitrogen-fixing *Rhizobium trifolii*, it seems reasonable to assume that energy is the limiting factor in the production of cyanoglucosides. The biosynthetic pathway of linamarin and lotaustralin is well known (Hughes and Conn 1976). It is identical for both cyanoglucosides, the only difference being the starting product: the lotaustralin pathway starts with leucine; the linamarine pathway, with valine (Butler and Butler 1960). Chew and Rodman (1979) have calculated the cost of producing one mole of lotaustralin from isoleucine as 2 mol ATP, 1 mol $NADH_2$ and 1 mol glucose, in addition to the cost of isoleucine. The pathway for linamarine is the same, I will therefore assume that the costs of producing linamarin and lotaustralin are identical. The total cost (including that of the amino acids) in terms of ATP is 109 mol or 5,476 kJ for 1 mol or 247.2 g linamarine.

From fresh weight determinations of the linamarin content of adult plants and the fresh weight/dry weight relation, I calculate the linamarin concentration to be 21 mg/g dry weight. The mean dry weight of the leaves is 11.7 g, so we have 245 mg linamarine per plant at a cost of approximately *5 kJ*. The best estimate of the caloric value of the flower heads is 18.8 kJ/g. The difference in flower production between *Acac* and *acac* plants is 6.9 g, which accounts for approximately *130 kJ*.

The difference in flower production thus far exceeds the need for energy to produce the linamarin/lotaustralin found in the plant. Is this discrepancy proof that there is no energy-based relationship between cyanoglucosides and reproductive yield? Certainly not, as some costs have not been taken in account, viz the cost of transport and the cost of maintenance. Very little is known about the cost of transport, but it is unlikely that the transport from the cytoplast to the vacuole will account for the difference we found. The maintenance cost of a metabolite is mainly dependent on its turnover rate. Again, very little is known on the turnover of secondary plant substances, but there are indications that linamarin is metabolized in the seedlings of *Hevea brasiliensis* (Lieberei et al. 1985). As maintenance costs are a permanent expenditure, in contrast to the one time cost of production, it may well be the former more than the latter that influences growth characteristics. A second possibility is that *Ac* is tightly linked to a gene or genes influencing flower production. A study of the evolution of the cyanogenic system in *T. repens* and the related *T. nigrescens* has provided some evidence for the location of the *Ac-ac* alleles on chromosomes that are only partly homologous. If this assumption is correct, it provides a logical explanation for the high amount of (pseudo) pleiotropic effects of *Ac* found by myself and other authors (Table 1).

However, much work has to be done before the actual relationship between *Ac* and flower production is clear. While there can be no doubt that there is a relation between the two (Daday 1965; Foulds and Grime 1972; Dirzo and Harper 1982b; present work), it is the magnitude of the effect that is of primary interest. If the cost of a chemical defense system in general would be much higher than assumed from the production cost, a cost-benefit analysis becomes important. Very few of these studies have been published. Hanover (1966) found that in white pine, growth is negatively correlated with monoterpene content; Van den Berg and Matzinger (1970) showed that in ten varieties of tobacco leaf, production is inversely related to nicotine content; Coley (1986) found a negative correlation between leaf production and tannin content in *Cecropia peltata*. Berenbaum et al. (1986) have shown that in the wild parsnip (*Pastinaca sativa*), variation in resistance to the parsnip webworm (*Depressaria pastinacella*) was 75% attributable to the variation in four furanocoumarins. As the resistance factors show a negative genetic correlation with potential seed production, the authors argue that in this case of a co-evolved plant and herbivore, an evolutionary "stalemate" has been reached. Although the parsnip-parsnib webworm system differs in many respects from the white clover-snail system, the conclusion that the production of protective substances limits the potential reproductive fitness is the same. Recently, Simms and Rausher (1987) failed to detect a cost of resistance of *Ipomoea purpurea* to one of its major herbivores (*Chaetocnema confinis*). As the mechanism of resistance is not known, these results seem less convincing.

The question arises: what is the significance of the results in this study for the study of the forces that maintain the cyanogenic polymorphism in *T. repens*? It is clear that the results cannot be directly extrapolated to natural populations. The restricted genetic background and the lack of correspondence between the experimental setup and the situation in natural populations prevents such an approach. The backcross used in the present study, segregated for a number of genes I was able to study, as morphological markers and isozyme genes, and undoubtedly segregated for many more that were not studied. Nevertheless, the nested ANOVA of the *Ac* effect on reproduction showed that *Ac* had a significant influence, whereas other genetic effects were not significant. My conclusion is, therefore, that *Ac*, or a gene (genes) tightly linked to the former, has an effect both on seedling survival and on sexual reproduction. How these effects influence the fitness of their bearers is dependent on a number of factors, both genetic and environmental, and the possible consequences of these interactions will be discussed at a future time. The most important conclusion is that *Ac* (and possibly *Li*), apart from its influence on the cyanogenic system, has another effect: whether it acts directly or indirectly on the energy balance in the plant, it must be looked upon as a cost item in the chemical defense system.

Apparently, the gene frequencies for *Ac* (and *Li*) found in natural populations depend on the way the potential cost and benefit of the cyanogenic system influence the fitness of the different cyanotypes.

In regions where molluscs are active in the period that white clover seedlings are present, plants with cyanoglucosides have a large advantage. Where moluscs are only predators of adult plants, the advantage is less, but may still be present. In any case, the advantage is partly or completely offset by the difference in flower production. In white clover, the influence of flower and seed production on fitness is dependent on the degree of sexual reproduction. In dense vegetation, asexual reproduction by stolons may be prevalent, whereas in open vegetation or in situations where disturbances are common, sexual reproduction may be more important. The synchronization of mollusc activity and seedling emergence may be responsible for the large scale cline in the frequency of *Ac* (and *Li*) observed in Europe by Daday (1954a). The difference in frost resistance between cyanotypes that has been regarded by Daday (1965) as the cause of the clines was not found in this study.

Other factors like a prevalence of predators that show selective eating and vegetation structure may be responsible for the pronounced differences in the frequency of *Ac* and *Li* between populations in one climatic region (Kakes 1987). The maintenance of the *Li* polymorphism is less clear. Linamarase is necessary for the protection against herbivores that do not have active β-glucosidases in their gut; that *Ac* and *Li* give protection when present together is not only shown by direct experiments, i.e. Dirzo and Harper (1982a), but also by the presence of strong linkage disequilibrium of *Ac* and *Li* (Ennos 1982; Kakes 1987).

The results reported in this study apply to plants not constrained in their growth by intra- or inter-specific competition. Experiments now started, with the same genotypes in dense stands mixed with grasses, will hopefully shed some light on the effect of *Ac* and *Li* on competition.

Acknowledgements. The author wishes to thank R. Reynders for cooperation with the grazing experiments and D. Hoonhout for typing the manuscript. L. Sanne prepared the figures and Dr. J. A. C. Verkleij made valuable comments on an earlier draft of the paper.

References

Berenbaum MR, Zangerl AR, Nitao IK (1986) Constraints on chemical coevolution: Wild parsnibs and the parsnib webworm. Evolution 40:1215–1228

Boersma P, Kakes P, Schram AW (1983) Linamarase and β-glucosidase activity in natural populations of *Trifolium repens*. Acta Bot Neerl 32:39–47

Burdon JJ (1980) Intraspecific diversity in a natural population of *Trifolium repens*. J Ecol 68:717–735

Butler GW, Butler BG (1960) Biosynthesis of linamarin and lotaustralin in white clover. Nature 1987:780–781

Chew FS, Rodman JE (1979) Plant resources for chemical defense. In: Rosenthal GA, Janzen DH (eds): Herbivores. Academic Press, New York, pp 271–307

Coley PD (1986) Costs and benefits of defense by tannins in a neotropical tree. Oecologia 70:238–241

Daday H (1954a) Gene frequencies in wild populations of *Trifolium repens* L. I. Distribution by latitude. Heredity 8:61–78

Daday H (1954b) Gene frequencies in wild populations of *Trifolium repens* L. II. Distribution by altitude. Heredity 8:377–384

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

Dirzo R, Harper JL (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, Harper JL (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

Dommée B, Brakefield PM, Macnair MR (1980) Differential root growth of the cyanogenic phenotypes of *Trifolium repens*. Oecol Plant 1:367–370

Ennos RA (1981a) Manifold effects of the cyanogenic loci in white clover. Heredity 45:127–132

Ennos RA (1981b) Detection of selection in populations of white clover. Biol J Linn Soc 15:75–82

Ennos RA (1982) Association of the cyanogenic loci in white clover. Genet Res 40:65–72

Foulds W, Grime JP (1972) The response of cyanogenic and acyanogenic phenotypes of *Trifolium repens* to soil moisture supply. Heredity 28:181–187

Hanover JW (1966) Genetics of terpenes I. Gene control of monoterpene levels in *Pinus monticola* Dougl. Heredity 21:73–84

Horrill JC, Richards AJ (1986) Differential grazing by the Mollusc *Arion hortensis* Fér. on cyanogenic and acyanogenic seedlings of the white clover, *Trifolium repens* L. Heredity 56:277–281

Hughes MA, Conn EE (1976) Cyanoglucoside biosynthesis in white clover (*Trifolium repens*). Phytochemistry 15:697–701

Hughes MA, Stirling JD (1982) A study of dominance at the locus controlling cyanoglucoside production in *Trifolium repens*. Euphytica 3:477–483

Hughes MA, Stirling JD, Colinge DB (1984) The inheritance of cyanoglucoside content in *T. repens*. Biochem Genet 22:139–151

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 Neerl 36:59–69

Lieberei R, Selmar D, Biehl B (1985) Metabolization of cyanogenic glucosides in *Hevea braziliensis*. Plant Syst Evol 150:49–63

Maher EP, Hughes MA (1973) Studies on the nature of the *Li* locus in *Trifolium repens*. Biochem Genet 8:113–126

Phillipson J (1964) A miniature bomb calorimeter for small biological samples. Oikos 15:130–139

Simms EL, Rausher MD (1987) Costs and benefits of a plant resistant to herbivory. Am Nat 130:570–581

Till-Bottraud I, Kakes P, Dommée B (1988) Variable phenotypes and stable distribution of the cyanotypes of *Trifolium repens* in Southern France. Oecologia Plant (in press)

Van den Berg P, Matzinger DF (1970) Genetic diversity and heterosis in *Nicotiana*. III. Crosses among tobacco introductions and flue cured varieties. Crop Sci 10:437–440

Ware WM (1925) Experiments and observations on forms and strains of *Trifolium repens*. J Agric Sci 15:47–67