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A Genetic Polymorphism for Tannin Production in *Lotus corniculatus* and Its Relationship to Cyanide Polymorphism

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Summary. A study of 172 unnamed populations and 22 cultivars of *Lotus corniculatus* showed: (1) that all plants of most strains of both categories contained leaf tannins (total 172 strains); (2) that 6 strains were tannin-negative; and (3) that 16 strains were polymorphic. Because of the small number of tested plants per strain, the above frequency of polymorphism is probably underestimated. Tannin-negative or polymorphic strains are frequent in Iran and Turkey. Leaf-tannin production is inherited as a monogenic dominant with tetrasomic inheritance. Repeated scores suggest that some individuals always, others sometimes and yet others never produce leaf tannins. Mean tannin content of 6 cultivars was strongly negatively associated with mean cyanide content.

Key words: Condensed tannins – Polymorphism – Inheritance – Cyanide – *Lotus corniculatus*

Introduction

Genetic polymorphisms have long attracted attention because of their evolutionary importance (Ford 1964). Each phase of a polymorphism occurs in a frequency too great to be attributed to a balance between recurrent mutation and selection, and it appears that each phase has a selective advantage under some conditions, but is at a disadvantage under others. Among plant polymorphisms, that for cyanide production has been much studied, especially in the forage legumes *Trifolium repens* and *Lotus corniculatus.* For both species it has been shown that the acyanogenic form is preferred as food by some invertebrates (Jones 1962; Crawford-Sidebotham 1972; Angseesing 1974), but that this form has a compensating advantage because of its greater cold tolerance (Daday 1954; Brighton and Horne 1977; but see also Ellis et al. 1977). In this paper we report on a new polymorphism for *Lotus corniculatus,* namely for tannin production in the leaves. We report genetic studies and show that mean tannin content of six cultivars is inversely related to mean cyanide content. Tannins react with soluble plant and animal proteins to form insoluble complexes (Jones and Mangan 1977). This affinity for proteins seems to explain the evolutionary importance of tannins also. When larvae of the winter moth (*Operophtera brumata*) were raised on a casein-rich diet, growth was reduced by the addition of oak-leaf tannins (Feeney 1968). Such tannins inhibited the hydrolysis of proteins by trypsin (Feeney 1969), and tannins inhibited fungal cellulase and pectinase also (Bell et al. 1965). It seems probable that at least in some small monogastric animals tannins render dietary proteins unavailable, thereby constituting a protection for the plant. The inhibition of cellulases and pectinases suggests that tannins may protect against fungi. Recent evidence has also shown tannins in the roots of *Lotus* species; this may be an important plant factor controlling effectiveness of the *Lotus/Rhizobium* symbiosis (Pankhurst and Jones 1980). The corresponding advantage of tannin-free plants is not known, but we present some evidence that it may consist in their greater resistance to cold or drought.

The term "tannin" has been used very loosely in the literature. In the present paper we define tannins as flavanol polymers, noting that such polymers act as protein precipitants. Substances which are so widespread among plants and which are so highly reactive with proteins are likely to have a considerable biological importance.

Materials and Methods

Table 1 gives the origin of 204 strains received under the name *Lotus corniculatus* L. The plants were grown outdoors in a field, using a randomised design. Each strain consisted of a row of 5 plants, with a spacing of 60 cm between plants and rows. In most strains all 5 plants were present, but the results are given here for all strains having at least 3 survivors. 22 of the strains were named varieties, all of which were assumed to be of cultivated origin. Of the unnamed strains, of known geographical origin, 172 were plant introductions kindly supplied by the NE-9 Regional Plant Introduction Station, Geneva, New York, (PI numbers ranging from 157531 to 358997), and these, together with the remaining 10 unnamed strains, were considered to be of non-cultivated origin. In addition, 50 plants of each of 6 cultivars were grown in a glasshouse, and tannin and cyanide content were measured for each individual plant. Three cultivars were chosen because of their high tannin content ('Viking', 'Maitland' and 'Franco'), and three for their low tannin content ('Empire', 'Winnar' and 'Leo').

Inheritance studies were made with *Lotus corniculatus.* The ovule parent of any cross is always given first. Two tannin-negative *Lotus* plants of cv. 'Winnar' from the randomised design, numbers 31-3 and 31-4, were intercrossed, and 31-3 was crossed to a high-tannin plant of cv. 'Vega', number 65-1. These parents, and all F_1 plants used as parents, were strongly self-incompatible and repeated observations on all parents always gave consistent tannin scores. Crosses were made by isolating pairs of plants in small cages, proof against large insects, and pollinating with washed bees. F_1 seed from the cross of 31-3 and 65-1 was taken off the tannin-negative parent only, and all F_1 plants were tannin positive.

Tannin content was estimated using a vanillin-HCl reagent (Jones et al. 1973). The basic scale used was zero for no tannins; 1 for a trace; 2 for a slightly stained leaf imprint; 3 for moderately stained; and 4 for intensely stained. However, because leaf imprints were sometimes intermediate between categories they were scored in half categories e.g. 1.5 for trace to slight. Apart from flavanol polymers (tannins) which are biologically active in precipitating proteins, false positive reactions could be obtained by monomeric flavanols, dihydrochalcones and anthocyanins (Sarker and Howarth 1976). Of these only anthocyanins could cause misleading results, since during numerous extractions of *Lotus corniculatus,* dihydrochalcones have not been detected and the monomeric fraction of flavanols has never amounted to greater than 8% of the total flavanol. In our experience, staining caused by anthocyanins was rather infrequent and confined to the tip of the petiole or to leaflet veins. During the later part of this study, imprints stained in this way but not in the main body of the leaflet were scored as negative.

Cyanide content was measured by the method of Joseph and Gaur (1971) and expressed in parts per million wet weight.

Plants grown in the randomised design were scored in the early autumn. Previous experience with several species suggested that tannin production is higher in summer or early autumn than in winter (Feeney and Bostock 1968; Cope et al. 1971; Ross and Jones 1974), so that the absence of tannins in the negative plants was probably usually due to inability to produce tannins, and not to unsuitable environmental conditions. Plants grown in the glasshouse were recorded in the summer or autumn, and some plants were recorded for tannins again in the winter. Facultatively tannin-positive plants of *L. pedunculatus* Cav. (synonym *L. uliginosus* Schkuhr) had previously shown continuous tannin production under glasshouse conditions (Ross and Jones 1974), so that it is likely that here too tannin production was favoured. Glasshouse plants were tested for tannins and for cyanide on the same or on successive days (4 cultivars), or were tested for tannin in early summer and for cyanide 4–6 weeks later ('Empire' and 'Winnar'). These last two cultivars were tested for both tannin and cyanide earlier in the season than were the other four. The results of Ellis et al. (1977) suggest that cyanide production is likely to be favoured under warm conditions in the glasshouse.

For the inheritance studies most plants were grown outdoors. Tannin-positive plants from the backcross *Lotus* progenies were low in tannins, and were scored up to five times under conditions favouring tannin production.

Statistical analyses were carried out using PDP 11/70, B 6700 and ZX 81 computers.

Results and Discussion

The Tannin Polymorphism

Table 2 gives the tannin results for 194 of the strains received as *Lotus corniculatus* and grown outdoors. Unlike Table 1, Table 2 excludes the 10 diploid strains (7 tannin negative, 3 positive) found by Forde and de Lautour (1978) to have only 12 chromosomes instead of the 24 chromosomes characteristic of *L. corniculatus.* (The eleventh diploid strains of these authors, PI 251149, was already excluded since it was received as *L. tenuis*). Table 2 gives the cultivated strains first, and gives also the geographical origins of the tannin-negative and the polymorphic noncultivated strains. We note that 19 of the cultivated strains (86.4%) contained only tannin-positive plants, and these include such well-known cultivars as 'Cascade', 'Viking', and 'Maitland'. Two cultivated strains (9.1%) had only tannin-negative plants ('Empire' and 'Winnar'), and one (4.5%) was polymorphic ('Taborsky'). A maximum of 172 of the noncultivated strains are truly *L. corniculatus.* Table 2 shows that 153 of these (89.0%) were entirely tannin positive, 4 were negative (2.3%) and 15 were polymorphic (8.7%). In 13 of the 15 polymorphic strains, negative plants were in a minority, with an overall ratio for the 15 strains of 49 tannin-positive plants: 21 negative, yielding 30.0% negatives. Since there was a maximum of 5 plants per strain, it is probable that the number of polymorphic strains has been underestimated. For example, it follows from the binomial theory that the probability of mis-classifying as tannin-positive a polymorphic strain with a true frequency of 30% tannin-negatives is 17% for a sample size of 5. For the same sample size, but with a true frequency of 5% negatives, this probability becomes 77%. This underestimate of the frequency of polymorphic strains does not of course undermine the finding of this paper that tannin polymorphism occurs in *L. corniculatus*; on the contrary, it is likely that the polymorphism is more widespread and important than the data show.

The geographical origin of the negative and polymorphic *corniculatus* strains is also of interest. Tables 2 and 3 show that the negative strains come from Iran and Turkey (2 strains each), and for the polymorphic strains (ignoring records from Australia and the United

Table 1. Origin of the experimental material received under the name *Lotus corniculatus* and grown in the randomised design

Name of cultivar or strain, or geographical origin
(1) *Named varieties (total 22)*
Empire, Viking, Maitland, Tana, Cascade, Winnar, Granger, Leo, Franco, Quimey, Ginestrino, El Boyero, Sao Gabriel, Sao Gabriel Type, Gulzower Hornklee, Rosnovsky Krag, Malejovsky, Viglassky, Taborsky, Yaki szarvakerep, Gelsvis, Vega
(2) *Geographical origin of the 182 unnamed strains*
Afghanistan (3 strains), Argentina (2), Australia (5), Austria (1), Brazil (10), Britain (1), Bulgaria (3), Chile (1), Czechoslovakia (4), Denmark (7), Egypt (1), Ethiopia (3), France (14), Germany (5), Greece (2), Hungary (4), Iran (6), Israel (3), Italy (16), Korea (1), The Netherlands (6), Norway (1), Poland (3), Portugal (4), South Africa (1), Spain (4), Sweden (5), Switzerland (10), Turkey (9), Uruguay (7), USA (12), USSR (21), Yugoslavia (7)

Table 2. Tannin production in *Lotus corniculatus* grown outdoors. Cultivated strains are given first in each column, and geographical origins are given for non-cultivated strains in columns 2 and 3 only

All tannin +	All tannin −	Polymorphic
Cascade	Empire	Taborsky
El Boyero	Winnar	Australia (1 strain)
Franco	Iran (2 strains)	Iran (3)
Gelsvis	Turkey (2)	The Netherlands (1)
Ginestrino		Sweden (1)
Granger		Switzerland (1)
Gulzower Hornklee		Turkey (3)
Leo		USA (2)
Maitland		USSR (3)
Malejovsky		
Quimey		
Rosnovsky Krag		
Sao Gabriel		
Sao Gabriel type		
Tana		
Vega		
Viglassky		
Viking		
Yaki szarvaskerep		
153 unnamed strains		

States, where the species is introduced) we find that the same two countries are represented by 3 strains each, as is the USSR. The countries of western and central Europe in the tables have only one strain each, and it appears that tannin-negative plants occur frequently in western and perhaps central Asia. Tables 1, 2 and 3, show that the number of uncultivated strains of *L. corniculatus* which contain negative individuals, as a proportion of the number of strains studied from a given country of interest, are: 5/6 for Iran, 5/7 for Turkey and 3/21 for the USSR. For western and central Europe these ratios are: 1/6 for the Netherlands, 1/5 for Sweden and 1/9 for Switzerland. Most European countries had no tannin-negative individuals, and it is quite striking that this should be the case for France and Italy, for example, where a total of 29 strains were studied. Turkey and Iran are known for their very cold winters and often hot dry summers, and it seems that one or both of these factors may be responsible for maintaining the polymorphism. We have already noted that winter cold could be responsible for maintaining the cyanide polymorphism in *Lotus* and *Trifolium*.

The experiment was not designed to reveal any conditional polymorphisms, such as are found in some strains of *L. pedunculatus*, where tannin polymorphism is detectable only under winter conditions. We shall see (below) that some individuals of *L. corniculatus* produce tannins facultatively, whereas others appear to produce them at all times. Evidence from breeding studies shows that some plants have never been found to produce tannins, despite repeated tests. Consequently this species has three types of plants: those which always, those which sometimes and those which never produce tannins.

Several other matters of technique may affect the results. Because the method is only semi-quantitative, it is sometimes difficult to classify plants which produce very small quantities of tannins. The cultivars 'Empire' and 'Winnar' initially appeared to be tannin free (Table 2), but later more intensive studies (Table 4) showed that in addition to negative plants both cultivars had some tannin-positive individuals, and may be regarded as polymorphic. However, no positive individual in 'Empire', and only occasional individuals in 'Winnar', had more than a trace of tannins. It may be that very small quantities of tannins give no significant protection to the plant, (e.g. there is insufficient tannin to precipitate the leaf proteins) so that a cultivar such as 'Empire', may be regarded as chemically polymorphic but biologically monomorphic. This situation is only a particular case of the dependence of any chemical result on the suitability and sensitivity of the method (Flück 1963; Jones 1972).

Tannin and Cyanide Production

The tannin and cyanide contents of the six cultivars that were studied more intensively are given in Table 4. Variation in cyanide content has previously been studied by many authors, e.g. Dobeš and Juza (1972), but there appears to be no previous study of both tannin and cyanide variation in this species. The table reveals polymorphisms for tannin in the cultivars

Table 3. United States Plant Introduction (P.I.) numbers for all non-cultivated tannin-negative and polymorphic strains, and for the three diploid tannin-positive strains. Origins and available chromosome numbers are also given. All plants were received as *Lotus corniculatus*[a]

Origin	P.I. no.	Chr. no.	Tannin score	Origin	P.I. no.	Chr. no.	Tannin score
Afghanistan	221937	12	–	Australia	316266	n.a.	±
Egypt	250571	12	–	Iran	227512	n.a.	±
France	[b]	12[f]	–	Iran	227849	24	±
Greece	[c]	12[f]	–	Iran	230348	n.a.	±
Iran	222193	24	–	Netherlands	226797	n.a.	±
Iran	228286	n.a.	–	Sweden	283624	n.a.	±
Korea	273443	12[d]	–	Switzerland	234808	n.a.	±
Portugal	[e]	12[f]	–	Turkey	204586	24	±
Turkey	205292	12[f]	–	Turkey	206447	24	±
Turkey	279944	24	–	Turkey	230332	n.a.	±
Turkey	304523	24	–	USA	314845	n.a.	±
Spain	253419	12	+	USA	314846	n.a.	±
Switzerland	234807	12	+	USSR	285281	n.a.	±
Turkey	206896	12	+	USSR	315455	n.a.	±
				USSR	325379	24	±

[a] Chromosome numbers from Forde and de Lautour (1978) (n.a. = not available)

[b] Supplied by CSIRO, Australia, no. CPI 28866

[c] Supplied from Larissa, Greece, No. M 6934

[d] Determined also by Dr. E. A. Wernsman (pers. comm.) and identified as *L. japonicus* by Dr. W. F. Grant (pers. comm.) and by Forde and de Lautour (1978)

[e] Supplied from Elvas, Portugal no. E 2649

[f] Identified as *L. tenuis* (Forde and de Lautour 1978)

+ = tannin positive, – = negative, ± = polymorphic

Table 4. Mean tannin and mean cyanide content of 50 plants in each of six cultivars of *Lotus corniculatus.* Measurements were made on an arbitrary semi-quantitative scale of 0 – 4 for tannins, and were in ppm wet weight for cyanide

Cultivar	Tannin content		Polymorphic[a]		Cyanide content		Polymorphic[a]	
	Mean ± SE	Range			Mean ± SE	Range		
'Franco'	2.69 ± 0.12	1.5 – 4	No	0%	274 ± 15.5	0 – 520	Yes	8%
'Maitland'	2.64 ± 0.13	0.5 – 4	No	0%	242 ± 13.6	0 – 380	Yes	4%
'Viking'	2.53 ± 0.14	0 – 4	Yes	4%	215 ± 10.5	72 – 415	No	0%
'Leo'	1.22 ± 0.14	0 – 3.5	Yes	24%	348 ± 16.4	190 – 680	No	0%
'Winnar'	0.47 ± 0.10	0 – 2.5	Yes	64%	614 ± 13.7	380 – 860	No	0%
'Empire'	0.30 ± 0.07	0 – 1	Yes	70%	745 ± 16.3	500 – 1000	No	0%
All plants*	1.64 ± 0.08	0 – 4	Yes	27%	407 ± 13.0	0 – 1000	Yes	3%

[a] Whether polymorphic, and proportion of negatives

* The regression coefficient among all plants is – 105.38, which is highly significant (< 1%), and demonstrates a strong negative association between tannin and cyanide contents

'Viking' and 'Leo', although in the small samples grown in the field only tannin-positive plants were found (Table 2). Table 4 shows also that the cultivars fall into three tannin groups: 'Franco', 'Maitland' and 'Viking' have a high tannin content, 'Leo' has a low tannin content, and 'Winnar' and 'Empire' are very low. Differences in mean tannin content between 'Viking' and 'Leo', and between 'Leo' and 'Winnar', are significant ($P < 0.1\%$). When the tannin test was repeated for the 197 surviving individuals of four cultivars under glasshouse conditions in the winter, the results showed that all tannin-negative plants remained negative, and that all positive plants of 'Franco' and 'Viking', and most of 'Leo' and 'Maitland', remained positive. Seven plants of the last two cultivars, however, were positive in the summer test but negative in winter, which is similar to the result found previously in *L. pedunculatus.* Table 4 shows that the two cultivars which are very low in tannins, 'Winnar' and 'Empire', are very high in cyanide, and the six cultivars show a strong tendency for

Table 5. Backcross data for leaf tannins in *Lotus corniculatus*

Cross[a]	Offspring		Ratio	P for		
	+	−	(+/−)	3:1	3.5:1	5:1
31−3(−) × 31−3−1(+)	110	19	5.8	<0.01	<0.05	0.7 −0.5
31−3−1(+) × 31−3(−)	100	22	4.5	0.1−0.05	0.3−0.2	0.7 −0.5
Total for above cross	210	41	5.1	<0.01	<0.05	0.95−0.99
31−3(−) × 31−3−9(+)	88	22	4.0	0.3−0.2	0.7−0.5	0.5 −0.3
31−3−9(+) × 31−3(−)	84	22	3.8	0.5−0.3	0.8−0.7	0.3 −0.2
Total for above cross	172	44	3.9	0.2−0.1	0.7−0.5	0.2 −0.1
31−3(−) × 31−3−12(+)	91	15	6.1	<0.01	<0.05	0.5 −0.3
31−3−12(+) × 31−3(−)	21	6	3.5	0.8−0.7	0	0.5 −0.3
Total for above cross	112	21	5.3	<0.05	0.1−0.05	0.8 −0.7
Total for 31−3 as ovule parent	289	56	5.2	<0.01	<0.01	0.9 −0.8
Total for 31−3 as pollen parent	205	50	4.1	<0.05	0.5−0.3	0.3 −0.2
Grand total	494	106	4.7	<0.01	<0.01	0.7 −0.5

[a] Ovule parent is given first
+ = tannin positive, − = tannin negative

high mean tannin content to be associated with low mean cyanide content. There was a highly significant negative regression coefficient (−105.38) for tannin and cyanide contents, for the 600 plants taken together. When the cultivars are arranged in order of mean cyanide content, the differences are statistically significant between all adjacent cultivars, except between 'Franco' and 'Maitland', and between 'Maitland' and 'Viking'. It would be interesting if the negative association between cyanide and tannin is also found in natural populations.

Inheritance of Tannins

The F_1 from the cross between the two tannin-negative parents of *Lotus corniculatus*, 31-3 and 31-4, gave 44 negative plants and 3 plants which scored negative on one occasion but gave a trace of tannins on another. It seems reasonable to accept all 47 plants as tannin negative. The reciprocal cross gave 41 negatives and 5 doubtful scores (negative and trace) but again we accept all plants as negative. It is possible that in the early stages of this experiment some plants were misclassified because of the presence of traces of anthocyanins. Four open-pollinated offspring of 31-4 were clearly tannin positive, since all scored higher than trace. All 33 F_1 from the cross 31-3 negative × 65-1 positive also were unambiguously tannin positive, with scores of up to 4 (intense), and with a mean spring score of 2.83 (range 1.5 to 3.5), which is close to the score of 3 (medium) given by the positive parent 65-1 on that occasion. Apparently tannin production is dominant, or nearly so.

Table 5 gives the backcross data for three of these F_1 plants (31-3-1, 31-3-9 and 31-3-12) crossed reciprocally to their maternal parent 31-3. Despite a tendency towards reciprocal differences, there is no significant heterogeneity for the six results ($P = 0.1$–0.05). The table shows a significant overall difference from the 3:1 ratio expected for digenic disomic inheritance, or from the 3.5:1 ratio for monogenic tetrasomic inheritance with chromatid segregation (Dawson 1941; Bubar and Mir 1965). The ratio does not differ from that for tetrasomic inheritance with chromosome segregation. There is some evidence, obtained from plants which were each scored five times, that the number of tannin negative plants scored less than five times could be overestimated by about 27%. When allowance is made for this possibility, the adjusted overall backcross ratio (513+ : 87−) remains significantly distinct from a 3:1 or 3.5:1 ($P < 0.01$), but is not distinct from a 5:1 ($P = 0.2$–0.1), so that the hypothesis of tetrasomic inheritance with chromosome segregation remains intact. The three F_1 tannin-positive plants may be regarded as duplex for the dominant allele *T*, the negative parent as recessive *tttt*, and the original positive parent 65-1 as probably quadruplex with genotype *TTTT*, although triplex or even duplex genotypes are not altogether excluded for this parent. The low scores for tannin-positive backcross offspring (e.g. mean score 1.19, with range 0 to 3.5, for 262 scores for the cross 31-3 × 31-3-12) suggest a gene-dosage effect. Possibly other genes affect the production of tannin.

Some of the difficulties in classifying backcross progeny could be due to the environmental sensitivity of tannin production. Such sensitivity is typical of secondary compounds (Flück 1963), and is found also for cyanide production (Ellis et al. 1977).

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