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GENETICS OF CYANOGENESIS, CYANOGLUCOSIDE* AND LINAMARASE PRODUCTION IN THE LEAVES OF *LOTUS CORNICULATUS* L.

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Abstract

The suggestion that *Lotus corniculatus* plants of stable phenotype for leaf cyanognesis were triplex or quadruplex whereas plants with unstable phenotype were duplex or simplex was investigated. None of the plants tested was found to be other than simplex at loci segregating for the glucosides and/or for the associated β-glucosidase (linamarase). *Ac/ac* and *Li/li* alleles were found to be segregating in the 1:1 ratio expected from simplex parents, although there appeared to be non-random assortment of *Ac* from *Li*. The parent plant C/27 appeared to be nulliplex at both the glucoside and enzyme loci whereas A/13 was simplex at the glucoside locus and nulliplex at the enzyme locus. Precise genotypes could not be assigned to most of the other parent plants because of the confused results obtained from the crosses. The suggestion that stable plants were likely to be triplex or quadruplex was not confirmed, however, in the material used. Synthesis of linamarase was observed in 25 of the 40 (+–) progeny of the cross A/13 x C/27 (+ – x – –) when they were exposed to winter conditions in the field, suggesting temperature-sensitive regulation of linamarase biosynthesis in these progeny.

Introduction

Armstrong *et al.*, (1912, 1913) were the first to investigate cyanogenesis in *Lotus corniculatus* L. They found that both the cyanoglucosides, linamarin and lotaustralin and the corresponding β-glucosidase, linamarase, are essential for the liberation of free HCN from the damaged tissues of the plant. They also discovered that there was some variability of the expression of the cyanogenic phenotype even within the same plant. When Dawson (1941) studied the formal genetics of cyanogenesis he had difficulty over choosing suitable parents for his crosses because of the problem of phenotypic instability. In spite of this complication he showed that leaf cyanogenesis in the plant was dominant over acyanogenesis and he obtained the tetrasomic F_2 ratio of 35:1.

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There are four reasons, however, why we cannot be satisfied with Dawson's work, nor with later studies on the genetics of cyanogenesis in *L. corniculatus* (e.g. Bansal, 1971).

1. Dawson (1941) indicated that he did not have segregation at both the cyanoglucoside and the linamarase locus.

2. Dawson himself states (page 54) that he was studying the genetics of the cyanoglucoside, but Seaney & Henson (1970) believe that he was studying the genetics of the enzyme.

3. It appeared from Dawson's paper that the genetic system in *L. corniculatus* is similar to that in *Trifolium repens* L. and thus it is very difficult to know which aspect of the system he was actually studying.

4. Bansal (1966) definitely showed that the formation of linamarase was dominant in leaves with some form of tetrasomic inheritance at that locus. There is, however, some confusion over whether the data were showing chromatid (Bansal, 1966) or chromosome (Bansal, 1971) segregation. The cytological evidence (Dawson, 1941; Poostchi, 1959; Somaroo and Grant, 1972) shows that formation of quadrivalents is of rare occurrence as the chromosomes in *L. corniculatus* are small and the chiasma frequency is less than 2 per bivalent (Wernsman *et al.*, 1964). Thus chromosome segregation is the more likely situation in *L. corniculatus*.

Ellis *et al.*, (1977) investigated two natural populations of *L. corniculatus* at Wharram Quarry, North Yorkshire (England) and Porthdafarch Anglesey (Wales). They found that the leaves of plants at Wharram Quarry were monomorphic for glucoside production and polymorphic for linamarase production whereas the Porthdafarch plants were polymorphic for both characters. The majority of the Wharram plants had stable phenotypes, but those from Porthdafarch could be classified into two classes, stable and unstable. On the basis of these results and assuming the Hardy-Weinberg law to hold for these populations they postulated that instability in the phenotypic expression shown by the plants from the Wharram Quarry was associated with homozygosity or higher order heterozygosity i.e. their genotypes were quadruplex or triplex for the dominant cyanoglucoside allele. On the other hand plants with unstable phenotypes could be lower order heterozygotes i.e. their genotypes were duplex or simplex for cyanoglucoside and enzyme alleles. The stability of phenotype would be based, therefore, on allele dosage.

The experimental plants used by Ellis *et al.*, (1977) were still available. These plants had been screened very carefully over several years for phenotypic stability and so they appeared to be excellent material for studying the formal genetics of cyanoglucoside and linamarase production in *L. corniculatus*. Furthermore they could be used

to determine whether the allele dosage explanation of phenotypic instability was correct. The results of crossing some of these plants are described in this paper.

Materials and Methods

The plants used in this work had been collected as cuttings from wild populations at Porthdafarch in 1974 and established as 'stock' plants in the Botanic garden at Hull, England. Many of these plants had been used previously in experiments (Ellis *et al.*, 1977; Keymer & Ellis, 1978) and so advantage was taken of existing knowledge about their individual characteristics. The plants were cloned by taking stem cuttings and rooting them in a peat/sand/grit mixture in the mist unit.

The following plants were chosen for the investigation: three clones (A/8, A/36 and C/23) of stable phenotype, positive for both cyanoglucosides and linamarase (++), 2 clones (D/8 and E/6) of stable phenotype positive for cyanoglucosides only (+–), 2 clones (A/6 and A/13) of unstable phenotype for the cyanoglucosides only (+– ⇌ – –), one clone (C/32) of stable phenotype positive for linamarase only (–+) and three clones (A/16, A/38 and C/27) of stable phenotype negative for both the cyanoglucosides and linamarase (– –). The assumption that these clones were of stated phenotypes was based on repeated testing for cyanogenesis made over a period of 18 months at 2-monthly intervals by Ellis *et al.*, (1977) in which the recorded temperature ranged from 6°C to 38°C. It was confirmed before the final choice was made (1977) that the plants used for these studies were of the phenotypes stated. The methods of testing leaves for cyanogenesis have been given in detail elsewhere (Jones, 1966).

A group of 9 plants, comprising one female parent plant in the centre surrounded by 8 male parent plants at a distance of 0.5 m from each other, were used for each cross. Crosses were made by isolating these groups of one female and 8 male parent plants under insect proof cages. All the open flowers of both the female and the male parent plants were removed before they were covered with the cages. When sufficient fresh flowers had opened a bumblebee was placed in cage. The majority of *L. corniculatus* plants are highly self-sterile (Seaney, 1964) and so the female (seed) parent plants were not emasculated. However, to obtain an indication of the proportion of the seeds which might be produced due to selfing, all the female parent plants used in direct or reciprocal crosses were selfed by isolating them individually in the cages together with a bumblebee. To minimize the chance of their being contaminated with pollen of unknown origin the bumblebees used in the breeding programme were captured from Hull University Campus which is about 3 km away from the experimental field where the crosses were made; *L. corniculatus* plants were uncommon in this area. However, when a few plants were discovered they were uprooted long before the bumblebees were captured. The date of release of a bumblebee was recorded and 3-15 days later the cage was removed and the pollinated flowers were labelled. Pollinated flowers were easily recognisable because they

remain attached to the pedicel for longer than unpollinated flowers. They were also distinguishable by the growth of the young pod through the keel petals whether or not fertilization had been effected. Individual pods were harvested before they dehisced.

The details of the crosses made are given in Table 2. In addition seven of the clones used as female parents in the direct and reciprocal crosses A/8, A/13, A/36, A/38, C/23, C/27 and C/32 were also selfed in the summer of 1978.

Seed germination

One hundred seeds of each progeny of direct and reciprocal crosses were scarified on silicon-carbide abrasive paper and were germinated at a temperature of 21°C (day) and 15°C (night) on moistened filter paper placed in Petri dishes. Petri dishes, distilled water and filter papers were previously autoclaved at 2×10^5 N m^{-2} pressure for 15 minutes at 120°C. After germination the seedlings were given 16 hours of light per day from fluorescent and incandescent lights at a photon flux density of 180-270 μ moles m^{-2} s^{-1} (Incoll *et al.*, 1977). After 5 days the seedlings were transplanted into John Innes compost No. 2 in plastic trays (36 cm x 22 cm), each tray containing 40 seedlings. The trays were placed in a warm glass house at a minimum temperature of 15°C. At the age of 5 weeks leaves from the young plants were tested for cyanogenesis using the method of Jones (1966).

Results and Discussion

The data in Table 1 show that the clones used in the breeding programme were self-sterile when they were isolated with bumblebees under cages. Because seed production under selfing conditions was zero, it can be concluded that contamination of bees with extraneous pollen can be ignored. The results of the crossing show (Table 2) that not one of the parent plants is triplex (or quadruplex) at either locus. The χ^2_1 tests show that *Ac/ac* and *Li/li* loci are segregating in the 1:1 ratio expected for the simplex condition, although there does appear to be non-random assortment of the *Ac* from the *Li* allele. There are, however, large numbers of progeny in phenotypic classes that could not have been expected from the known phenotypes of the parent plants. For example, the cross A/38 x C/32 (– – x –+) and its reciprocal gave rise to ++ progeny. The fact that the reciprocal crosses were made in different years and yet behave in essentially the same way rules out both selfing and pollen contamination as possible explanations. Thus we can conclude that it is not possible to use cyanogenesis as a genetic marker to estimate the degree of selfing of a – – plant when isolated with a ++ plant (e.g. Bansal, 1966). The most clear cut segregation is the progeny of the cross C/27 x A/13 (– – x +–) and its reciprocal. This leads to the conclusion that C/27 is nulliplex at both loci and A/13 is simplex at the cyanoglucoside locus and nulliplex at the enzyme locus. Having determined the genotypes of these plants it was hoped that they could be used to deter-

mine the genotypes of the parent plants in each of the other crosses in which they were involved. Unfortunately, this has proved to be impraticable. For example, cross A/16 x A/13 (– – x +–) give glucosidic to aglucosidic progeny in the proportion 44:43 (i.e. 1:1) as expected from a nulliplex x simplex, but there is immediate problem when we consider linamarase production. The enzyme had been detected in neither A/16 nor in A/13 during the previous 36 months and yet there are 31 progeny (out of 87) that contain the enzyme. The cross C/27 x A/13 (– – x +–) suggests that it is not A/13 which passes the ability to produce enzyme to its progeny and so we must conclude that A/16 is simplex at the enzyme locus. On the other hand there is totally contradictory evidence. Ellis *et al.*, (1977) showed A/13 to be unstable, undergoing transition from +– to – –. In the experimental field in December 1977 and January 1978, we found A/13 to be ++, becoming +– when brought into a warm glasshouse in January 1978. This result suggested that the C/27 x A/13 progeny should also be subjected to the field environment during the winter. Only the A/13 x C/27 progeny were available and unfortunately most of the 33 – – progeny were so small that there was insufficient material for satisfactory tests for cyanogenesis to be made after treatment. But of the 40 +– progeny, 25 became ++ when placed outside the glasshouse. Clearly there is temperature sensitive regulation of enzyme biosynthesis in the progeny and the ratio obtained suggests a simple genetic basis for this.

Table 1. Results of self-pollination of the clones used as the female parents in the breeding programme, using a 'caged' bumblebee as pollinating agent.

Clone No.	No. of pollinated flowers	No. of pods set
A/8	213	0
A/13	27	0
A/36	59	0
A/38	204	0
C/27	147	0
C/23	41	0
C/32	20	0

Table 2. The progenies obtained from test crossing Porthdafarch *L. corniculatus* with non glucosidic non-enzymic (– –) plants.

Cross			Seeds sown	Plants reaching testing stage	Phenotypes of progeny				$\chi^2_{(1)}$ *Ac/ac*	$\chi^2_{(1)}$ *Li/li*	Year in which the cross was made
					++	+–	–+	– –	1:1	1:1	
A/38 – –	x	A/8 + +	100	88	28	10	12	38	1.63	0.72	1977
A/8 + +	x	A/38 – –	100	97	34	8	17	38	1.74	0.25	1978
A/38 – –	x	C/23 + +	150	136	52	23	33	28	1.44	8.5**	1977
C/23 + +	x	A/38 – –	100	99	39	8	2	50	0.25	2.91	1978
A/38 – –	x	C/32 – +	120	103	24	2	10	57	–	2.18	1977
C/32 – +	x	A/38 – –	62	59	11	0	17	31	–	0.15	1978
C/27 – –	x	A/36 + +	100	97	16	28	24	29	0.83	2.97	1977
A/36 + +	x	C/27 – –	100	86	22	18	23	23	0.41	0.18	1978
C/27 – –	x	C/23 + +	100	94	32	21	12	29	1.53	0.38	1977
C/23 + +	x	C/27 – –	100	95	20	32	20	23	0.85	2.36	1978
C/27 – –	x	A/13 +– ⇌ – –	100	79	0	38	0	41	0.11	–	1977
A/13 +– ⇌ – –	x	C/27 – –	100	73	0	40	0	33	0.67	–	1978
C/27 – –	x	A/8 + +	100	52	16	7	14	15	0.69	1.23	1977
A/8 + +	x	C/27 – –	100	92	12	21	11	48	7.34**	23.00***	1978
C/27 – –	x	C/32 – +	100	94	21	2	43	28	–	12.29***	1978
C/32 – +	x	C/27 – –	69	38	10	0	12	16	–	0.94	1978
C/38 – –	x	D/8 + –	100	97	27	19	5	46	0.25	–	1977
A/38 – –	x	E/6 + –	100	90	26	12	3	49	2.17	–	1977

Table 2. (Contd.).

Cross	Seeds sown	Plants reaching testing stage	Phenotypes of progeny ++	+–	–+	––	$\chi^2_{(1)}$ *Ac/ac* 1:1	$\chi^2_{(1)}$ *Li/li* 1:1	Year in which the cross was made
A/38 x C/27 – – – –	95	87	13	14	5	55	–	–	1978
C/27 x A/6 – – +– ⇌ – –	100	39	2	22	2	13	2.07	–	1977
C/27 x E/6 – – +–	75	18	0	12	1	5	2.0	–	1977
A/16 x A/36 – – ++	100	90	29	12	5	44	0.71	5.37*	1977
A/16 x A/13 – – +– ⇌ – –	100	87	27	17	4	39	0.01	–	1977
A/16 x D/8 – – +–	100	91	34	15	15	27	0.53	–	1977
A/16 x E/6 – – +–	100	79	22	21	9	27	0.62	–	1977
A/16 x A/6 – – +– ⇌ – –	100	89	32	4	5	48	3.24	–	1977
A/36 x A/38 ++ – –	100	98	43	11	10	34	1.02	0.65	1978

* $0.05 > P > 0.01$ ** $0.01 > P > 0.001$ *** $P < 0.001$

These results make it almost impossible to assign a precise genotype to most of the parent plants in this crossing programme. There is, however, no evidence contradicting the conclusion that A/13 is simplex at the glucoside locus. Although all other crosses gave more confused results, there is no evidence that any of the segregations corresponded to the 3:1, 5:1 and ∞ :0 test-cross ratios expected from duplex, triplex or quadruplex parents respectively. We can conclude, therefore, that all the test parents are simplex for those loci under test. Consequently the suggestion by Ellis *et al.*, (1977) that the stable plants are likely to be duplex, triplex and quadruplex has not been substantiated. On the contrary, the situation seems to be much more complicated, but there does appear to be evidence of temperature-sensitive regulation of linamarase synthesis.

Assortment of *Ac* and *Li* alleles

On its own, the cross A/38 x A/8 (– – x ++) and its reciprocal suggests linkage between the glucoside and linamarase loci in A/8, but this is not confirmed by the cross C/27 x A/8 (– – x ++). Similarly A/36 x A/38 (++ x – –) suggests linkage, whereas A/36 x C/27 (++ x – –) does not. There is disequilibrium here, but there is no pattern, presumably because the distribution of phenotypes within a progeny is confounded with phenotypic instability. The same problem arises when considering the cross A/38 x C/27. These plants were chosen because they lacked both cyanoglucoside and linamarase (– –) and under normal circumstances a cross of this kind would be a test for allelism. The appearance of linamarase producing individuals in this progeny is partly explained from the observation that both parents became –+ in the experimental field during the winter of 1977-78. The presence of cyanoglucosides in 27 of the 87 progeny tested cannot be explained in a similar manner because the cyanoglucosides have not been detected in either of the parents under experimental field conditions.

If there happen to be two loci for cyanoglucoside production segregating in this cross, then one individual must be nulliplex at one locus and the second parent must be nulliplex at the other. This does not rule out the possibility that each parent can be heterozygous at the locus for which it is not nulliplex. For example, a cross between two aglucosidic plants could be *A a a a b b b b* x *a a a a B b b b*. Although the ability to produce the cyanoglucoside is dominant, that is an individual simplex for *A* and for *B* will be glucosidic, the phenotypic ratio expected in the progeny is 3 aglucosidic: 1 glucosidic. This ratio corresponds to the segregation of A/38 x C/27 (60 aglucosidic and 27 glucosidic χ^2_1 3:1 = 1.48). Furthermore, there are only two other crosses in Table 2 involving A/38 and C/27 in which the other parent is aglucosidic, i.e. A/38 x C/32 (– – x –+) and C/27 x C/32 (– – x –+) and the progeny segregated into 125 aglucosidic 27 glucosidic (χ^2_1 3:1 = 0.40) and 99 aglucosidic; 33 glucosidic (χ^2_1 3:1 = 0) respectively. These data fit the hypothesis.

But, if we accept that A/38 has the genotype *A a a a b b b b* and C/27 is *a a a a B b b b* then to give glucosidic progeny when crossed with A/38 and with C/27 the only appropriate genotype for C/32 is *A a a a B b b b* and such plant will be glucosidic. This difficulty could be overcome by postulating a third locus *C* that is nulliplex in C/32 and triplex in A/38 and C/27, but there are no independent data supporting this suggestion.

There is another possibility worth exploring. It has been assumed, following the results of Dawson (1941) and of Bansal (1966) that cyanogenesis, cyanoglucoside production and linamarase production are dominant. If cyanoglucoside production is recessive at Porthdafarch, the progeny from the crosses A/38 x C/27, A/38 x C/32 and

C/27 x C/32 are immediately explained as those expected from the crossing of simplex plants i.e. 3:1. Is there any evidence in Table 2 to show that this hypothesis is unlikely? It has already been explained that the majority of the segregations appear to be 1:1. The one cross that is significantly different from 1:1, C/27 x A/38 and its reciprocal (χ^2_1 1:1 = 7.1) is also significantly different from 3:1, (χ^2_1 = 14.81) but not from 2:1, (χ^2_1 = 2.0). There is, therefore, no evidence from the crosses presented in Table 2 to show that cyanoglucoside production may not be recessive in this material. It is unfortunate that the design of the experiments was based on the results obtained by Dawson (1941) and by Bansal (1966), i.e. test crossing to – – plants, and so no crosses of the type ++ x ++ or +– X +– were undertaken. Breeding from some of the progeny produced in the experiments reported above, for example intercrossing +– progeny of the cross C/27 x A/13, should indicate whether the dominance relationship between cyanoglucoside and acyanoglucoside alleles in the Porthdafarch population is different from elsewhere.

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