GENETICS OF CYANOGENESIS IN WHITE CLOVER (*TRIFOLIUM REPENS*)

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CONTENTS

The leaves of some white clover plants contain a cyanoglucoside which liberates minute quantities of hydrocyanic acid when hydrolysed in the presence of an enzyme. Cyanogenetic glucosides have been found in many other species. Plants containing relatively large amounts of these glucosides are often harmful, and even poisonous, to animals. It is known that some of the sorghums contain at certain growth stages sufficient cyanoglucosides to be injurious to the health of stock grazing them.

I. Previous investigations

Doak (1933, 1935) and Rigg *et al.* (1933) have shown by quantitative determinations that the potential HCN content of certain white clover forms is considerably in excess of the amount usually considered as lethal to farm stock. In view of this fact, strongly cyanogenetic forms such as New Zealand "mother seeds" and English wild white would be expected to be highly toxic, but, as pointed out by the New Zealand investigators, this is not the case,[1] since pastures consisting mainly of white clover with high potential HCN content are often grazed continuously for long periods by the same stock without the animals showing any apparent harmful effects. It has been suggested as an explanation of

[1] Evidence confirming the non-toxicity of a strongly cyanophoric form of English wild white was obtained in 1933 by the writer in an experiment specially designed to test this point.

the non-toxicity of these potentially high HCN forms that the glucoside is only partially hydrolysed during the digestive process, and that a part of the hydrocyanic acid liberated is inactivated before reaching the blood stream.

The occurrence of a cyanoglucoside in white clover was first reported by Mirande (1912). It was soon afterwards realized that some of the white clover agrotypes show very considerable differences in regard to their cyanophoric properties, and up to the present most of the investigations on cyanogenesis in white clover have been mainly concerned with the possibility of using this character as a means of distinguishing between the different forms. Armstrong *et al.* (1913) reported that all the English wild white plants which they tested were cyanophoric, while all the cultivated plants tested gave negative reactions. These results are not in exact conformity with those which have been obtained since by numbers of other workers. All the 100 English wild white samples, and seventeen out of 103 samples of cultivated white clovers tested by Pethybridge (1919) gave positive reactions. Sampson (1922) found that all the Dutch white, and 68 % of English wild white individuals tested were acyanophoric. Ware (1925) found that about 71 % of the wild white plants on Romney Marsh in Kent were cyanogenetic. Doak (1933, 1935) and Rigg *et al.* (1933) found a strong positive correlation between productive capacities of the various New Zealand forms and their HCN content.

The writer has recently carried out a fairly comprehensive study[1] of the cyanogenetic reactions of a large number of white clover agrotypes from various sources in Great Britain and abroad. As a result of these investigations, during the course of which over 30,000 individuals were tested, it was found that English wild white was extremely variable in respect to this character, some subtypes containing as few as 3 % cyanogenetic plants, others as many as 98 %. The cultivated, or the so-called "Dutch" white clover, and the wild form endemic to central and eastern Europe consisted almost entirely of acyanophoric types. In contrast to these, certain large-leaved wild forms growing in the western provinces of France consisted almost exclusively of strongly cyanogenetic plants. White clover is not endemic to North America, Australia or New Zealand, but was introduced into these regions probably from Europe within comparatively recent times. The forms now growing wild in these countries are extremely variable in regard to their cyanophoric proper-

[1] The results of these studies will be published shortly.

ties, a fact which seems to suggest that they were originally derived from several sources.

There seems to be no doubt that the present day cultivated strains of white clover were originally derived from various European wild forms, but we have no direct evidence, except in the case of a few recent forms, as to their precise origin. It is interesting to note that a certain amount of circumstantial evidence bearing on the point has been obtained from a comparative study of the cyanogenetic reaction of the wild and cultivated forms.

The investigations reported in this paper were conducted at the Welsh Plant Breeding Station during the years 1929–34: they deal exclusively with the mode of inheritance of cyanogenesis in white clover.

II. MATERIAL AND METHODS

The parent plants used in these studies were selected from English wild white, ordinary cultivated and New Zealand white clovers. All cross-pollinations were made by hand in glasshouses protected from insect pollinators: the flowers were not emasculated. Although white clovers are normally cross-fertilized, a considerable number of the plants are slightly self-fertile when artificially self-pollinated, the average seed setting from selfing being about 5 %. The writer (1931)[1] has shown that self- and cross-sterility in white clover are governed by a series of multiple allelomorphs acting as oppositional factors. Since pollen tubes carrying "unlike" sterility alleles are capable of growing down the styles more rapidly than those with "like" alleles, it would be expected that the number of seeds resulting from selfing would be considerably less than 5 % when the plants are outcrossed. Indirect evidence that this is actually the case has been obtained from several out-crosses in which the female parents were recessive and the male parent dominant for simple distinctive characters such as certain types of leaf-markings. Apart from a few cases in which the female parents were relatively highly self-fertile, only an occasional plant resulting from selfing appeared in these out-crosses. Although there is no doubt that a small amount of unavoidable self-fertilization does sometimes occur during cross-pollination, in the vast majority of cases the number of selfed individuals which appear among progenies derived from compatible crosses are far too few to invalidate the results obtained.

The seedlings were raised under glass; most of them were transplanted

[1] Further and more comprehensive data relating to this problem have been obtained since that paper was published.

direct into 4½ in. pots, in which the plants were tested, but a few families were also planted in the field at 30 in. apart. Unless the ground is known to be free from "hard" seeds of white clover, the latter procedure is not recommended on account of the possibility of contamination. It may be mentioned in this connexion that an occasional volunteer plant appeared even among the pot cultures, despite the fact that only sterilized soil was used.

The cyanogenetic reactions of the plants were tested by the Guignard's picrate paper method described by Armstrong *et al.* (1913). Some of the first results obtained by this method proved to be unsatisfactory, and on that account have been omitted from this paper. It was found that duplicate tests sometimes gave conflicting results. In view of these inconsistencies, which were most noticeable in weakly cyanogenetic progenies, it was decided to carry out a number of preliminary experiments on the best method of conducting these tests before proceeding further with the main investigations.

It was soon observed that the intensity of the reaction on the picrate paper rapidly diminished with the age of the leaves. By far the strongest reactions were obtained by using very young folded leaves; even fully expanded one-day-old leaves sometimes showed marked diminution in their reaction, while old leaves of proved cyanogenetic plants sometimes had no perceptible effect on the picrate paper. The reactions of leaves of different ages collected on the same day from four cyanogenetic plants are shown below:

Plant no.	Few weeks old	Few days old	Young folded leaves
1	Very weak	Medium	Very strong
2	Weak	Medium	Strong
3	None	Very weak	Very strong
4	Weak	Strong	Very strong

Another series of preliminary tests was undertaken to determine the number of young leaves required to give a full reaction. One leaf was generally sufficient to change the colour of the picrate paper to fairly distinct red, but at least three to four leaves were necessary to give what was regarded as the maximum reaction. The addition of toluene, or a similar substance capable of rapidly killing the plant cells, appears to be essential for the complete hydrolysation of the white clover cyanoglucoside, since in its absence no HCN was produced even by strongly cyanogenetic leaves. One drop of toluene was found to be quite as effective as larger quantities.

It was also observed that the addition of very small amounts of water to the leaves accelerated fermentation to a slight degree.

Incubator temperatures of 20–25° C. gave quite satisfactory results in most cases, but weakly cyanophoric material gave somewhat stronger reactions when incubated at temperatures of 35–45° C.

As a result of these observations the following method of testing for the presence of HCN was finally adopted. From four to six very young leaves were selected from each plant and placed at the bottom of a $2 \times \frac{1}{2}$ in. glass tube, to which one drop of toluene was added. In dry seasons, the plants were thoroughly watered before the leaves were collected. A freshly prepared narrow strip of picrate paper was suspended in the tube, and held in position by a well-fitting cork. The tubes, together with the necessary controls, were then placed for about 40 hr. in an incubator with a temperature of about 40° C. The liberation of HCN by the leaves was usually clearly indicated by the change in the colour of the picrate paper from bright yellow to various shades of red, ranging from faint red to a deep brownish red.

The cyanogenetic plants were classified according to the intensity of their reactions on the picrate paper into five classes, namely, very weak, weak, medium, strong and very strong, which probably corresponded in a general way with the amounts of HCN produced.

III. INHERITANCE OF CYANOGENESIS

Considerable differences were observed between certain families in regard to their cyanogenetic reactions. In many families, particularly those derived from certain New Zealand white clover plants selected on account of their high HCN content, all the cyanophoric individuals gave strong, or very strong, positive reactions, but in certain other families all the positive plants gave uniformly weak, or medium, reactions, while in other progenies some of the sister plants showed consistently wide variations in the amount of HCN produced. Tests on a few sister plants giving widely different reactions were repeated several times over a period of 2 to 3 years. Although the intensity of the reactions varied within certain limits according to the season, being usually most intense in the spring, the results obtained from any given plant were, on the whole, remarkably consistent, and there seems to be no doubt that the extreme variation in the HCN content of young leaves of different plants was due less to differences in environmental conditions than to some intrinsic property of the individual. It is suggested that these inter- and intra-family differences are due to the presence of modifying factors acting as intensifiers or diluents.

(1) *Crosses between homozygous cyanogenetic and acyanogenetic plants.* Eleven F_1 progenies derived from crosses between acyanophoric individuals and plants which were subsequently shown to be homozygous for cyanogenesis were tested. The results are summarized in Table I.

TABLE I

Cyanogenetic reaction of white clover plants resulting from **AcAc** × **acac** *crosses*

Pedigree no.	No. of families	Cyanogenetic reactions		Total plants tested
		Positive	Negative	
47	2	211	—	211
57	3	399	—	399
160	1	46	—	46
209	1	75	—	75
266/7	2	400	1	401
267/70	3	363	—	363
Total	11	1494	1	1495

As seen from Table I, with one exception, all the 1495 plants derived from crosses between individuals giving positive and negative cyanogenetic reaction gave a positive reaction. The solitary acyanophoric individual which appeared in these crosses was manifestly a rogue, for it was quite different in type from any of the other plants in the cross in which it was found. In view of the results obtained it is evident that cyanogenesis in white clover is dominant over the acyanogenetic character.

(2) *Crosses between acyanogenetic plants.* Twenty crosses were made between individuals giving negative reactions. As shown in Table II, with the exception of one individual which was probably a rogue,[1] all the 2474 F_1 plants resulting from these crosses gave a negative reaction, showing that acyanophoric parents were true-breeding in respect of this acyanophoric character.

(3) *Backcrosses.* Data from thirty-two backcrosses are presented in Table III.

As seen from Table III, the data for thirty-one out of the thirty-two backcross families are in reasonably close agreement with the 1 : 1 ratios expected on the basis of simple Mendelian segregation, the differences between the observed and calculated frequencies as shown by the P values being less than errors expected from random sampling. The remaining family, namely, 267 (1), gave results which did not conform with the expected equality, and at present no explanation can be advanced to account for the great excess of cyanophoric plants in this

[1] This plant was probably derived from a "hard" seed, dormant in the soil.

TABLE II

Results of cyanogenetic tests on plants derived from crosses between acyanophoric parents (acac × acac)

Pedigree no.	No. of families	Cyanogenetic reactions: Positive	Cyanogenetic reactions: Negative	Total plants tested
12	1	—	120	120
34	1	—	113	113
36	13	1	1944	1945
140	3	—	237	237
152	1	—	39	39
365	1	—	20	20
Total	20	1	2473	2474

TABLE III

Segregation for cyanogenesis in backcrosses (Acac × acac)

Family no.	Cyanogenetic reactions: Positive	Cyanogenetic reactions: Negative	Total plants tested	χ^2	P
34 (2) 2 × 68	45	49	94	0·1702	0·69
35 (2) 191 × 233	30	37	67	0·7312	0·49
35 (2) 201 × 221	12	18	30	1·2000	0·28
35 (2) 241 × 251	22	25	47	0·1914	0·68
35 (2) 248 × 251	25	30	55	0·4544	0·50
36 (2) 48 × 51	7	9	16	0·2500	0·64
36 (3) 238 × 273	57	51	108	0·3333	0·58
36 (3) 266 × 289	133	124	257	0·3150	0·60
36 (3) 278 × 254	101	101	202	0·0000	—
36 (3) 297 × 254	125	124	249	0·0040	0·95
36 (3) 297 × 304	72	84	156	0·9226	0·35
36 (3) 302 × 289	53	50	103	0·0872	0·78
36 (3) 310 × 289	70	69	139	0·0070	0·94
36 (3) 312 × 269	10	13	23	0·3912	0·55
45 (2) 16 × 18	35	27	62	1·0322	0·32
46 (2) 12 × 18	142	110	252	4·0062	0·05
154 (1)	115	132	247	1·1700	0·29
155 (1)	64	74	138	0·6328	0·45
161 (1)	24	22	46	0·0868	0·78
266 (1)	41	38	79	0·1138	0·75
372 (1)	39	37	76	0·0526	0·82
373 (1)	73	84	157	0·7706	0·40
374 (1)	48	43	91	0·2746	0·62
267 (1)	166	119	285	7·7508	>0·01
267 (2) 2 × 4	80	73	153	0·3202	0·59
375 (1)	33	31	64	0·0624	0·80
376 (1)	34	34	68	0·0000	—
377 (1)	44	46	90	0·0444	0·84
378 (1)	19	19	38	0·0000	—
269 (1)	93	98	191	0·1308	0·71
380 (1)	24	27	51	0·1764	0·69
381 (1)	86	89	175	0·0514	0·82
Total observed	1922	1887	3809	21·7334	
Calculated (1 : 1)	1904·5	1904·5			

backcross. A total of 3809 plants was tested, and of this number 1922 gave positive reactions and 1887 negative. Here again there is a very close agreement between the observed and expected frequencies assuming a 1 : 1 segregation.

(4) *F_2 and F_3 crosses.* Some of the F_1 plants used for backcrossing were also intercrossed. The data for the resulting F_2 families together with those for two F_3 families are shown in Table IV.

TABLE IV

Segregation for cyanogenesis in F_2 and F_3 crosses (Acac × Acac)

Family no.	Generation	Cyanogenetic reactions: Positive	Cyanogenetic reactions: Negative	Total plants tested	χ^2	P
34 (2) 1 × 2	F_2	32	8	40	0·5333	0·48
36 (3)	F_2	86	35	121	0·9944	0·33
81 (2) 20 × 84	F_2	192	68	260	0·1845	0·68
141 (2) 8 × 10	F_2	145	57	202	1·1154	0·30
266 (2) 4 × 5	F_2	89	31	120	0·0444	0·84
267 (2) 7 × 8	F_2	41	15	56	0·0952	0·77
267 (2) 2 × 7	F_2	171	55	226	0·0530	0·82
268 (2) 5 × 6	F_2	48	16	64	0·0000	0·99
268 (2) 4 × 5	F_2	85	28	113	0·0029	0·99
268 (2) 1 × 5	F_2	25	4	29	1·9424	0·18
269 (2) 2 × 5	F_2	88	30	118	0·0112	0·93
36 (3) 297 × 294	F_3	116	44	160	0·5333	0·48
36 (3) 297 × 302	F_3	101	33	134	0·0098	0·93
Total observed		1219	424	1643	5·5198	0·94
Calculated (3 : 1)		1239·75	413·25	1643		

As seen by reference to Table IV the observed distributions for each of the eleven F_2, as well as for the two F_3 families were in close accordance with the expected 3 : 1 ratio on the assumption that there is only a single factor difference between cyanophoric and acyanophoric plants. The total number of F_2 and F_3 plants tested was 1643, of these 1219 were cyanogenetic and 424 were acyanogenetic, an excess of only 10·75 in the recessive class over expectation.

IV. Summary

1. The presence and absence of cyanoglucoside in white clover are governed by a simple pair of factors which are designated **Ac** and **ac**.

2. The cyanophoric character is completely, or almost completely, dominant to the acyanophoric.

3. The marked differences which occur between certain families, and in some cases between plants within the same families, in regard to their HCN content are probably due to modifying factors acting as intensifiers or diluents.

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