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THEORETICAL AND APPLIED GENETICS

Leaf Mutants in Diploid Red Clover (*Trifolium pratense* L.)

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Summary. Leaf mutants were isolated and genetically stabilised in diploid red clover (*Trifolium pratense*). The major alteration showed normal trifoliolate leaves changed into multifoliolate leaves composed of 4-, 5-, 6- and 7 leaflets. As a result of recombination with other mutant alleles several genotypes were isolated with a different mode of leaf setting, different shapes and sizes of leaflets, variations in the whole plant habit, etc. A careful description was made of the mutant morphology and the development and genetic background was estimated as (h^2_{sl}). The mutants demonstrated no disturbances in their generative reproduction and as a rule set seeds better than the standards. The primary evaluation permits a conclusion that the leaf alteration (complexity) is governed by at least three recessive pairs of alleles of additive action. The phenotypic expression of the altered leaves depends simply on the number of recessive alleles. Apart from the above there were some modifying genes of incomplete penetration.

Key words: *Trifolium* – Mutant – Multifoliolate – Petiolulate – Heritability

Introduction

Characteristic sets of morphological features and a continued generative reproduction of plant populations separate individual taxons and form an orderly arrangement of variation in nature. Mutations and their multidirectional expressions are capable of bringing about profound deviations from the well-balanced arrangement of plant characters during the process of evolution (Halisz 1972; Schmalhausen 1975).

In the above context changes in the leaf structure in clover constitute an interesting phenomenon. They go far beyond the so far adopted basic taxonomic criteria for characters for many other species, constituting a valuable material for genetic analysis and a potential of variation for breeding purposes.

Materials and Methods

The mutants were isolated from diploid hybrids of American and Canadian varieties ('Lakeland' and 'Dallard') belonging to the botanical variety *expansum* ('Haussk') crossed with Polish agroecotypes belonging to the botanical variety *sativum* subvar. *praecox* (Witte). The studies were initiated in 1963 and the heritable type established over ten consecutive years by sibmatings under insulators with bumble-bees. The major feature of the mutant forms was an alteration in the leaf structure from trifoliolate into multifoliolate leaves (ml) with 4-, 5-, 6- and 7 leaflets. Minor alterations were characterised by a changed structure of other leaf features, e.g. shape and size of leaflets, sessile leaflets, petiolulate leaflets, length of leaf stalks and altered plant habit. The studies were designed to analyse the morphology and development of mutant clover forms as compared to the standards (three Polish cvs, viz. 'Gloria', 'Hruszowska' and 'Skrzeszowicksa'). Observations and measurements were made over the years 1972-1976. Altogether 25 mutant strains were analysed in a partially balanced square lattice design, in three replications according to Cochran and Cox (1950).

Heritability coefficients (h^2_{sl}) for particular mutant leaf types were calculated using the formula:

$$h^2_{sl} = \frac{MS_K - MS_E}{MS_K + (b - 1)\, MS_E}$$

where

h^2_{sl} – heritability sensu lato

MS_K – mean square for object

MS_E – mean square for error

b – number of replications [Barcikowska 1970].

The variance was calculated by accepted statistical procedures.

Results

The F_2 hybrids of the American and Canadian diploid red clover crossed with local agroecotypes revealed plants with multifoliolate leaves prevailing in the rosette and normal trifoliolate leaves present on generative shoots. Twelve plants were selected and propagated by sibmatings and severe negative selection was applied to plants with normal leaves, not only with respect to rosette stages but to generative shoots as well. As a result stabilised mutant forms were obtained in the F_{10} and propagated in the space-isolated field.

Mutant forms were primarily characterised by alterations in leaf structure. Their leaves were multifoliolate (Fig. 1). The number of leaflets per leaf ranged from 4 to 7. Leaves with five leaflets prevailed. Besides the major alterations, considerable differences were found in the anizofilia and heterofilia of the leaflets (Figs. 2-4).

The consecutive generations revealed plants with leaf structure much more altered showing leaflets set on petioles (Fig. 1). These were designated by the symbol pll (petiolulate leaflets) to differentiate them from sessile leaflets (Pll). Further generations demonstrated leaves with cup-leaflets (Fig. 5).

Plants with altered leaf structure displayed a wide variation range both in plant habit and type (Fig. 6). The differentiation was initiated in the rosette stage and resulted from different lengths of leaf stalks. Modifications in the rosette became more pronounced with phenotypic penetration of other genes, viz. erect leaflets changed into drooping, convex and prominent forms which are shown in Figs. 7, 8, 9 and 10. Extreme cases showed plants so conspicuously different from the *Trifolium* type (Fig. 10) that was not possible to classify them to recognised taxons until flowering. Apart from the above, alterations in morphotypes proceeded in the period of generative growth and following maturity (Fig. 11).

The rough mutant leaves set above cotyledons were composed of single leaflets, their edges formed a smooth margin, they were either round or ellipse-shaped and they

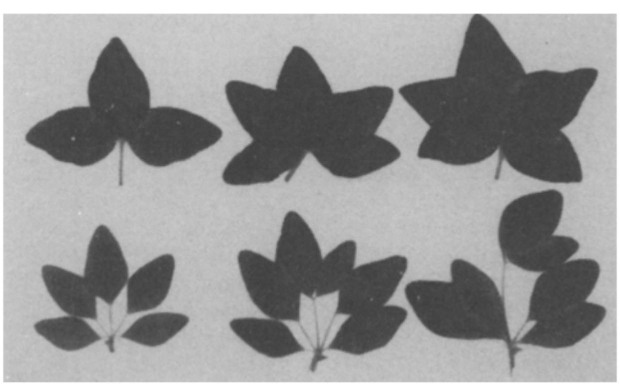

Fig. 1. Red clover leaves. On the left of top row: normal leaf and multifoliolate sessile leaves (ml Pll); bottom: multifoliolate petiolulate leaves (ml pll)

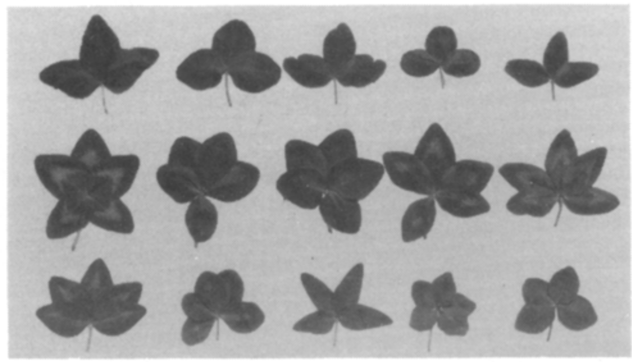

Fig. 2. Variation in size and shape of leaves and leaflets. Upper row: leaves of control plants; middle and bottom row: mutants ml Pll

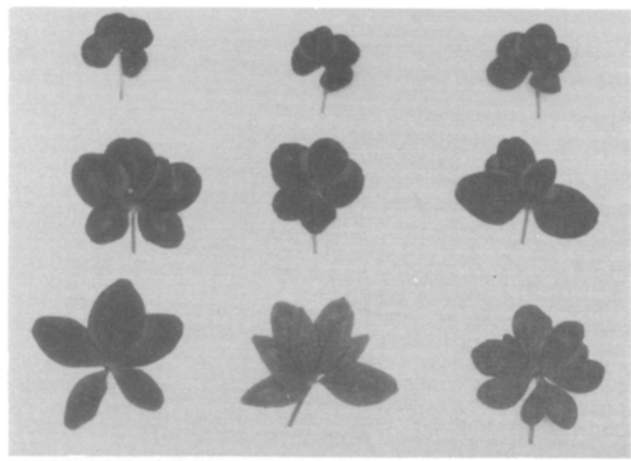

Fig. 3. Variation in leaf and leaflet structure of mutants

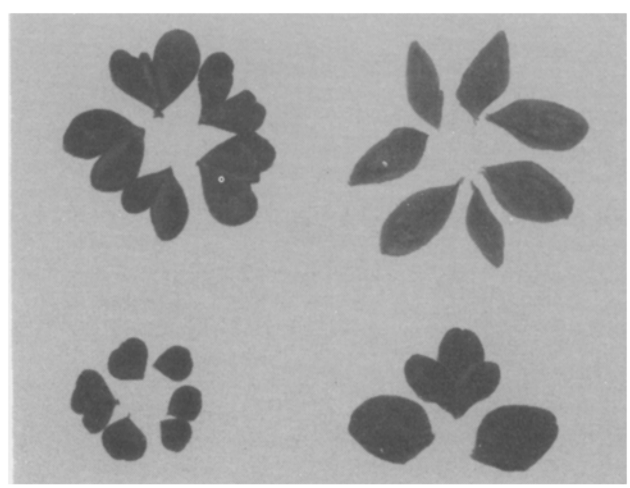

Fig. 4. Separate leaflets from some leaves in Figure 3 showing the compound structure and shape

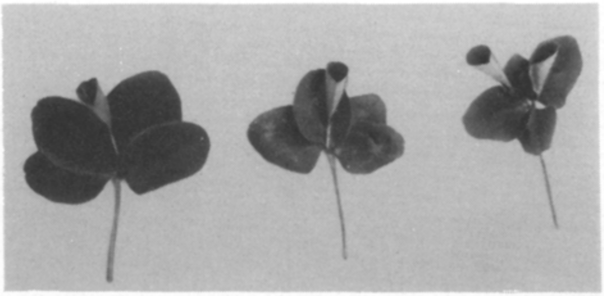

Fig. 5. Multifoliolate mutant leaves exhibiting some leaflets changed into cup-leaflets

Fig. 6. Alteration of single mutants in a rosette stage

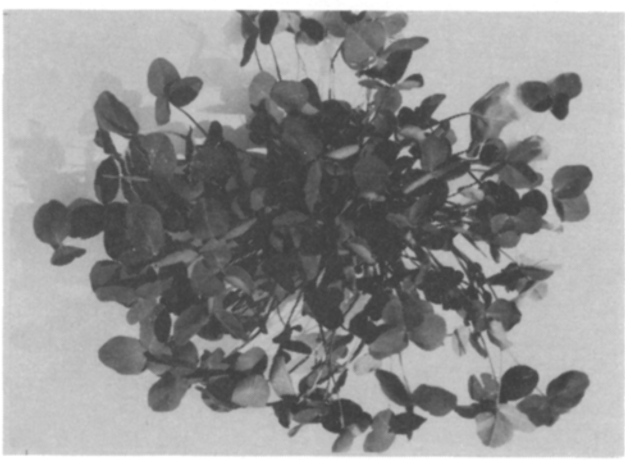

Fig. 7.

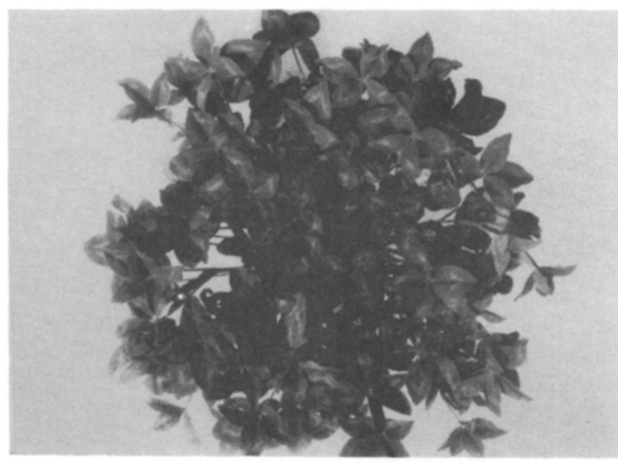

Fig. 8.

Fig. 9.

Fig. 10.

Fig. 7-10. Plants in rosette stage. Fig. 7 – Controls; Figs. 8-10 – plants with mutated leaves

Fig. 11. Mature mutant plants

did not differ from controls. Not until the true leaves were developed did the alterations become visible. The variation range of complex leaf structure is shown in Table 1. The five-leaflet leaves prevailed in number, as indicated.

Range of variation in the leaf surface of multifoliolate mutants is shown in Table 2. The mutated true leaves were characterised by having larger leaf surfaces than the trifoliolate leaves of standards. However, the total leaf surface per plant varied. The mutant with sessile leaflets (Pll) had a total leaf surface per plant larger than the standard while the petiolulate mutant (pll) had the total leaf surface smaller, despite the larger leaflet surface per leaf. The above resulted from a differentiated number of leaves per plant in the two mutants. Analysis of variance for the rosette leaf surface, for the fifth leaf on generative shoot and for the total leaf surface per rosette revealed a significant difference between the mean values for multifoliolate leaf mutants Pll and pll and for mutants and standards. These characters were insignificant between standard varieties. Genetic control of leaf types and small effect of environment on phenotypic variation of characters results from high heritability coefficients (Table 3). They are shown separately for 1973-1976, due to extremely varying weather conditions in these seasons.

The drastic mutations of phenotype called for analysis of the overall plant vigour as well as for generative repro-

Table 1. Variation in leaf types in diploid red clover (1973-1976)

Mutants	with 3 leaflets			with 4 leaflets			with 5 leaflets			with 6 leaflets			with 7 leaflets			$LSD_{0.05}$
	$\bar{x} \pm s_{\bar{x}}$	Range	Coefficient of variance	$\bar{x} \pm s_{\bar{x}}$	Range	Coefficient of variance	$\bar{x} \pm s_{\bar{x}}$	Range	Coefficient of variance	$\bar{x} \pm s_{\bar{x}}$	Range	Coefficient of variance	$\bar{x} \pm s_{\bar{x}}$	Range	Coefficient of variance	
No. of leaves in rosette stage																
ml pll	–	–	–	4.1 ± 0.1	1-16	10.2	17.3 ± 0.1	6-39	23.9	2.4 ± 0.1	1-8	4.7	1.5 ± 0.2	1-5	4.0	1.73
ml Pll	–	–	–	13.8 ± 0.2	4-38	29.8	43.3 ± 0.2	12-89	43.7	9.4 ± 0.2	1-16	8.6	4.7 ± 0.3	1-9	5.2	2.12
No. of leaves on generative stems																
ml pll	16.2 ± 0.2	1-25	14.18	13.2 ± 0.1	5-19	10.6	44.8 ± 0.3	20-70	20.1	2.6 ± 0.2	1-6	7.3	1.4 ± 0.1	1-4	3.8	1.50
ml Pll	1.4 ± 0.1	1-5	4.9	15.8 ± 0.3	8-34	14.2	68.9 ± 0.4	28-92	37.6	7.8 ± 0.3	1-15	12.9	2.7 ± 0.1	1-7	4.9	2.91

Table 2. Variation in leaf surface in diploid red clover mutants (1972-1976)

Character	Mutant ml pll			Mutant ml Pll			Controls		$LSD_{0.05}$
	$\bar{x} \pm S_{\bar{x}}$	Range	% Controls	$\bar{x} \pm S_{\bar{x}}$	Range	% Controls	$\bar{x} \pm S_{\bar{x}}$	Range	
Rough leaf surface (cm^2)	1.1 ± 0.05	0.8- 1.3	114.6	0.9 ± 0.01	0.7- 1.1	93.8	0.96 ± 0.1	0.8- 1.2	0.24
Rosette leaf surface (cm^2)	51.8 ± 0.5	38.5- 69.1	170.9	45.2 ± 0.7	31.7- 56.8	149.2	30.3 ± 0.8	18.6- 38.6	2.56
Surface of single leaflet of rosette leaf	12.2 ± 0.1	8.2- 16.2	169.0	8.6 ± 0.2	6.1- 11.5	121.1	7.1 ± 0.3	4.5- 9.2	2.71
Surface of the fifth leaf on generative stem	43.9 ± 0.2	39.8- 50.6	170.2	37.3 ± 1.6	30.2- 45.7	144.5	25.8 ± 0.9	17.0- 34.6	3.97
Surface of single leaflet of leaf on generative stem	8.2 ± 0.1	6.8- 15.8	135.3	6.1 ± 0.1	4.6- 8.9	100.7	6.1 ± 0.3	3.4- 9.7	2.51
Surface of leaves in rosette stage	1798.3 ± 8.2	1238.0- 3143.6	83.2	2533.7 ± 10.1	1927.0- 3772.4	117.2	2160.0 ± 10.6	1424.5- 3628.4	203.16
Surface of leaves on generative stems	7056.1 ±	4238.5- 8797.5	77.8	9896.7 ± 13.1	6534.5-13584.5	109.2	9070.0 ± 12.5	6027.8-14936.1	283.34
Surface of leaves on single plant	8878.5 ± 12.3	7134.6-14368.7	79.4	12428.6 ± 14.6	8273.5-16427.5	111.0	11200.0 ± 15.8	7238.7-16248.6	257.46

Table 4. Variation in seed yield parameters in diploid red clover mutants (1972-1976)

Character	Mutant ml pll			Mutant ml Pll			Controls		$LSD_{0.05}$
	$\bar{x} \pm S_{\bar{x}}$	Range	% Controls	$\bar{x} \pm S_{\bar{x}}$	Range	% Controls	$\bar{x} \pm S_{\bar{x}}$	Range	
No. of heads per plant	47.5 ± 1.1	5 - 58	53.8	73.9 ± 1.6	14 -126	83.7	88.3 ± 1.2	57 -132	7.43
No. of florets per head	142.8 ± 1.5	68 -242	108.0	140.3 ± 2.1	64 -202	106.0	132.0 ± 2.1	75 -162	15.68
No. of seeds per head	82.3 ± 1.0	51 -128	105.0	94.2 ± 1.8	42 -182	120.0	78.3 ± 1.7	51 -142	6.31
Fertility (%)[a]	60.5 ± 1.6	10.7- 94.8	98.1	66.1 ± 1.9	25.3- 89.8	107.0	61.7 ± 1.3	47.0- 93.5	4.28
1000 seeds weight (g)	1.4 ± 0.1	0.6- 1.7	82.4	1.6 ± 0.2	0.9- 2.0	94.1	1.7 ± 0.2	1.6- 2.1	0.28
Yield of seeds per plant (g)	8.7 ± 0.3	2.0- 14.9	84.5	12.8 ± 0.9	3.5- 18.0	110.3	11.6 ± 0.5	6.5- 18.5	2.64

[a] Fertility was considered as a relative value of the number of seeds to the number of florets ratio

Table 3. Heritability coefficients (h^2_{sl}) of different types of leaves

Year	Mutants	Leaf complexity				
		3-foliolate	4-foliolate	5-foliolate	6-foliolate	7-foliolate
1973	ml pll	0.54	0.72	0.87	0.73	0.76
	ml Pll	0.56	0.76	0.84	0.70	0.71
1974	ml pll	0.66	0.83	0.83	0.76	0.72
	ml Pll	0.69	0.80	0.87	0.73	0.63
1975	ml pll	0.61	0.74	0.79	0.91	0.70
	ml Pll	0.57	0.68	0.77	0.79	0.62
1976	ml pll	0.71	0.70	0.68	0.73	0.71
	ml Pll	0.67	0.73	0.65	0.60	0.78
Mean	ml pll	0.63	0.75	0.79	0.78	0.72
	ml Pll	0.64	0.74	0.80	0.72	0.67

duction capacity. Not a single mutant form was found lethal or semilethal. Despite the considerable range of variation, the process of flowering was characteristic enough. As a rule, the beginning, full and the end of flowering were approximately 14 days earlier for Pll mutants and 14 days later for pll mutants than for standards.

No differences were found in the structure of head and florets between the mutants and controls. The number of heads per plant varied conspicuously. The range of variation of this character in the Pll mutant surpassed the variation of standard varieties. The number of florets per head varied respectively. The average was as follows: standard varieties – 131.7; multifoliolate mutant Pll – 140.9 and multifoliolate mutant pll – 142.8 (Table 4). Cytological analysis of meiosis and of pollen grain viability revealed not the slightest irregularity. Mutant fertility (seed set to flower number) was positively favourable and at the relative range 10.7-96.0% it was approximately 7.3% higher in the Pll mutant than in the standards (Table 4).

Due to various factors seed yield per plant ranged from 2.0 to 18.0 g. The Pll mutant had the highest mean yield (12.8 g) (Table 4). No differences were observed in the weight of 1000 seeds.

The regular generative reproduction provided extremely favourable grounds for the genetic research required in such cases (results of these investigations will be published in separate papers) as well as for establishment and selection of forms of positive selection value with a view to develop new cultivars within this species.

Discussion

Alterations in the leaf structure of red clover from trifoliolate to multifoliolate were commonly encountered in artificial tetraploids (Łaczyńska-Hulewicz 1956). In addition, Thaler and Weber (1955), in *Trifolium pratense*, and Stark (1926), in *T. repens*, reported seven-leaflet leaves. Analysis of unifoliolate forms of *T. repens* was described by Atwood (1938). No mutations of this type were reported in red clover. Extensive studies on the changeability of trifoliolate into multifoliolate leaves were made on alfalfa (*Medicago* sp.) (Bauder 1938; Makeyer 1940; Bingham 1965; Bingham and Murphy 1965; Ferguson and Murphy 1973). However, these were allotetraploid forms.

Heritable alteration of leaf structure characteristic of *Trifolium* genus in diploid red clover is intriguing both from the point of view of evolution and genetics. It is generally assumed that the pinnate leaves of *Papilionaceae* are of an ancestor character (v. Denffer et al.) 1962; Eams 1961). From these, palmate leaves can be derived (e.g. lupine) as well as trifoliolate (clover, alfalfa) and leaves with tendrils at their tips (peas) etc. Taking the multifoliolate leaf characteristic for a specific atavism, one can expect it to result from primitive and wild characters governed by dominant genes. However, the diploid red clover mutants isolated in the course of this study were of a recessive character. Considering the mutant phenotypic expression it can be assumed that they suggest reversion to primitive forms although their genesis is entirely different. They are the result of mutations of series of alleles in the direction from dominants to recessives.

Genetically, the phenomenon of multifoliolate leaves in clover (*T. pratense* and *T. incarnatum*) was interpreted as an effect of reversible mutations (Malinowski 1974). Taking the above for granted, these mutants cannot be genetically stabilised. The present studies demonstrated the multifoliolate leaf character in diploid clover to the heritable, thus the high frequency of occurrence of reversible mutations proved impossible.

The different interpretation of these two phenomena is likely to result from two factors: (i) red clover being an obligatory allogamous plant, i.e. highly heterogenic, (ii) the leaf structure alteration being a polygenic character.

Mutant alleles, the recessive in particular, are generally masked by normal alleles as a result of panmixis. Recessive alleles come into action sporadically as a result of random mating although the effects decline in subsequent generations and they finally become heterogeneous. Further, if we decide on analysing the character governed polygenically with an additive action, the phenotypic expression of mutant alleles is likely to vary and, at uncontrolled cross pollination, has a character of modified changes from generation to generation.

From crosses between forms of different botanic varieties plants with a considerable number of multifoliolate leaves were obtained in segregants. As a result of sibmating and strong negative selection of trifoliolate leaves in subsequent generations, the phenotypic expression of recessive alleles responsible for the complex leaf structure was increasingly more conspicuous until genetically stabilised forms with this character were obtained. Sofar, the genetic analysis indicates three pairs of alleles to be responsible for the leaf structure alteration. Sibmating caused gradual homogenisation of plant populations in several generations. The above facilitated identification of other recessive alleles, e.g. sessile or petiolulate leaf, shape and size of leaflets, plant habit, earliness etc. Thus, favourable grounds were provided for synthesising a wide range of conspicuously differentiated genotypes.

Extensive data on other plant species (Murawski and Blasse, 1954; Kulik 1943; Schwanitz 1952, 1953) indicate changes in leaf structure to produce considerable deviations from normal formation of flowers and from their functioning.

No correlations of this types were observed in red clover. Organs of generative reproduction developed same as standard forms, their fertility was comparable and sometimes surpassed that of standards.

The wide range of variation of multifoliolate mutant leaves in diploid red clover constitutes a valuable material for genetic studies in this species and moreover, a wide spectrum of variation for agricultural practice. The primary evaluation of some strains shows them to have positive selection value.

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