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Hybrids of *Trifolium pratense* L. (2n=14+2) with *T. diffusum* Ehrh. (2n=16) and other 16-chromosome *Trifolium* species

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Abstract. 16-chromosome forms of red clover (2n=14+2) were crossed to six *Trifolium* species with the chromosome number 2n=16. Hybrid plants were derived from the cross of a stable 16-chromosome red clover *T. pratense* with *T. diffusum* (2n=16). No seeds were obtained from reciprocal crosses. F_1 hybrid plants were morphologically more similar to *T. diffusum*, whereas their other characters, e.g. flower number per head, were intermediate between the species crossed. All F_1 hybrids had the chromosome number 2n=16. Meiosis proceeded with large irregularities. The average number of bivalents per cell was 3.32, and that of univalents – 9.98. Univalents exhibited a high stickiness and frequently formed "end-to-end" configurations and chains consisting of about a dozen of so chromosomes. Bivalents were straight. Lagging chromosomes and chromosome bridges were observed during AI; lagging chromosomes were also found during AII. After an equalizing division, tetrads and different from them microspore polyads were formed. 16-chromosome hybrids were male- and female-sterile. No hybrids were obtained from the stable 16-chromosome red clover *T. pratense* (n=7 chromosomes) crossed to the selected clover species (*T. apertum* Bobr., *T. alexandrinum* L. and others) with n=8 chromosomes.

Key words: *Trifolium pratense*, *Trifolium diffusum*, interspecific hybrids, sterility, meiosis.

Studies on spontaneous *T. pratense* forms with two accessory chromosomes (genomes with 2n=14+2) in individuals and lines derived from sib-mating were aimed at stabilization of this karyotype (STRZYŻEWSKA 1974, 1976, 1984). The genome with 16 chromosomes became stable in the F_{14} generation. That was indicated by seed setting and by the lack of secondary disomics in

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16-chromosome lines. Due to obtaining a new red clover karyotype an attempt was made in 1987-1993 to cross it to *T. diffusum* and other 16-chromosome species with the karyotype 2n=16. This work was carried out to elucidate to what extent the chromosomes of spontaneous 16-chromosome red clover (*T. pratense*) and 16-chromosome *T. diffusum*, *T. apertum*, *T. alexandrinum* and others are homologous and what is their affinity with a spontaneous 16-chromosome genome.

This paper presents results of the study on the somatic chromosome number, measurements of morphological traits, course of meiosis and fertility of males (pollen viability) and females (seed-setting after pollination) in the 16-chromosome hybrid of *T. pratense* with *T. diffusum*. Results of crossing red clover with selected 16-chromosome *Trifolium* species are also presented.

Material and methods

In 1987-1993, plants of red clover with 2n=16 chromosomes were crossed to 16-chromosome species: *T. diffusum*, *T. apertum*, *T. alexandrinum*, *T. hybridum*, *T. hybridum* ssp. *anatolicum* and *T. vavilovii*. Red clover was used as a mother plant. Crosssing was carried out under greenhouse conditions. Inflorescences of red clover plants were emasculated by hand – 20 flowers per head. Pollen of *T. diffusum* and other 16-chromosome clover species was transferred onto the stigmas of the mother plant pistils.

The fertility of males (pollen viability) and females (seed setting) in the first generation was studied in all the plants. The percentage of seed set in relation to the number of pollinated flowers in the heads was estimated. Pollen viability was determined by their stainability in Belling's mixture.

The somatic chromosome number was studied in the root tips. The collected roots were left in distilled water for 2 hours at the temperature of 1-3°C, fixed in the mixture of absolute alcohol and propionic acid (3:1), hydrolyzed in HCl at 60°C for 5 min. Root tips were stained in a 2% propino-carmine directly on a microscopic glass to make squashes. The course of meiosis was analysed on smears stained with aceto-carmine or propiono-carmine. Photographs were taken by a Nikon camera at magnification 100×10.

Morphological measurements of plants, i.e. shoot number, head number and flower number per head as well as shoot length, flower length and some traits of flowers, were made during the flowering stage.

Results

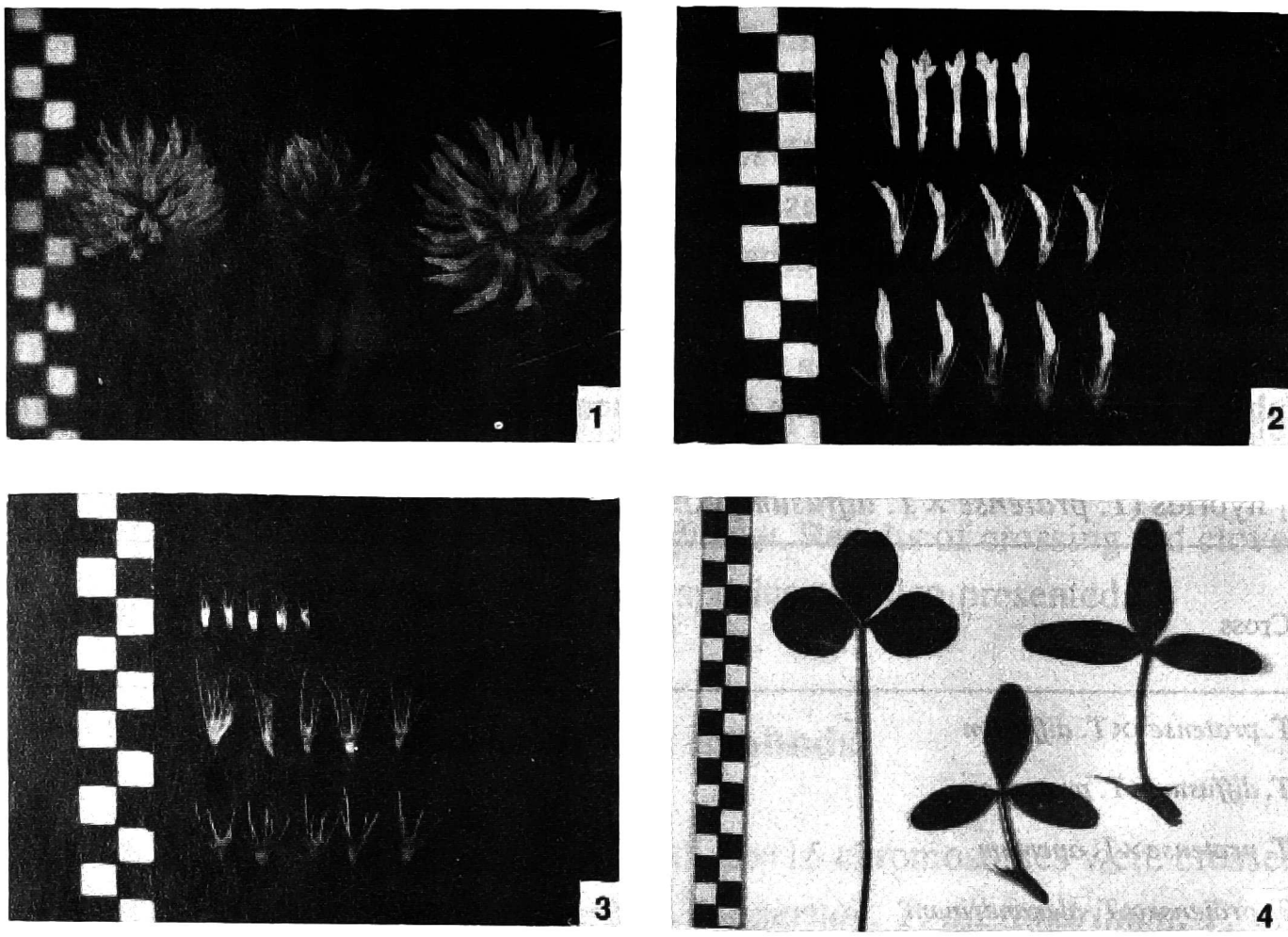
Pollination of 360 flowers of red clover (2n=16) with *T. diffusum* (2n=16) pollen resulted in obtaining 91 seeds (25.3%). They germinated equally well like seeds of the parental forms. All F_1 plants came to flower. No hybrids were obtained from the reciprocal cross *T. diffusum* (2n=16) × *T. pratense* (2n=16) (Table 1).

Table 1. Seed setting after crossing a spontaneous form of *T. pratense* (2n=14+2) with *T. diffusum* (2n=16) and with five other 16-chromosome *Trifolium* species, and in the F_1 hybrids (*T. pratense* × *T. diffusum*) pollinated with pollen of parental plants

Cross	No. of pollinated flowers	No. and % of mature seeds	No. of F_1 plants	Pollination
T. pratense[1] × *T. diffusum*	360	91 (25.3)	91	by hand
T. diffusum × *T. pratense*	220	0	0	"
T. pratense × *T. apertum*	880	3 (0.3)	0	"
T. pratense × *T. alexandrinum*	1648	40 (2.4)	0	"
T. pratense × *T. vavilovii*	1384	32 (2.3)	0	"
T. pratense × *T. hybridum*	1752	18 (1.0)	0	"
T. pratense × *T. hybridum* ssp. *anatolicum*	980	16 (1.6)	0	"
F_1 × *T. pratense*	1465	0	0	"
F_1 × *T. diffusum*	814	0	0	"
F_1 × *T. pratense*	4420	0	0	by insects

[1] *T. pratense* – a spontaneous form with 2n=14+2

The hybrid plants were compared to the parental forms in respect of their morphological traits. Hybrids were morphologically more similar to *T. diffusum*. They had one erect, poorly tillering stem and fine heads with loosely distributed flowers. With regard to the leaf blade index, length and pubescence of calyx sepals they were more similar to the paternal form, whereas flowers of F_1 plants were similar to those of the maternal form in size. With regard to other traits, e.g., flower number per head, they were intermediate between the crossed species. Generally, F_1 hybrids were less vigorous that the initial forms. Results of morphological measurements are summarized in Tables 2 and 3, in Figs. 1-4 heads, flowers, calyx sepals and leaf blades of hybrids and their parental forms are presented. The somatic chromosome number of F_1 plants was 16 (Fig. 5).



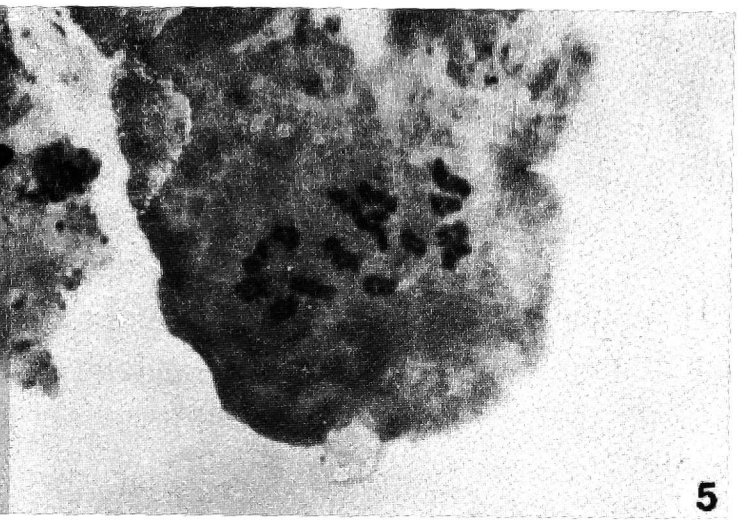
Figs. 1-4. Heds, flowers, calyx sepals and leaf blades of parental forms and F_1 hybrid (*T. pratense* 2n=14+2 × *T. diffusum* 2n=16)

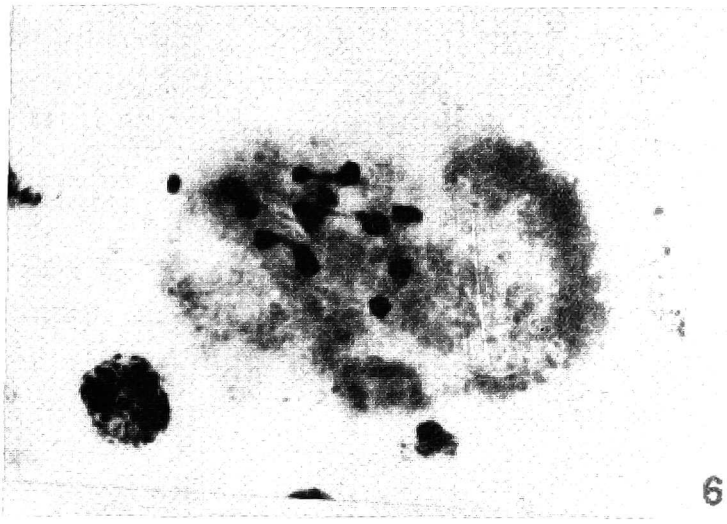
An analysis of pollen fertility showed no pollen grains with plasma. The examined plants were completely sterile and despite backcross pollination with pollen from both parental forms, attempts to obtain seeds failed (Table 1). Part of F_1 plants and 16-chromosome red clover were put outside the greenhouse to make possible their free pollination by insects. However, in spite of simultaneous plant flowering and insect activity at that time, no seeds were obtained (Table 1).

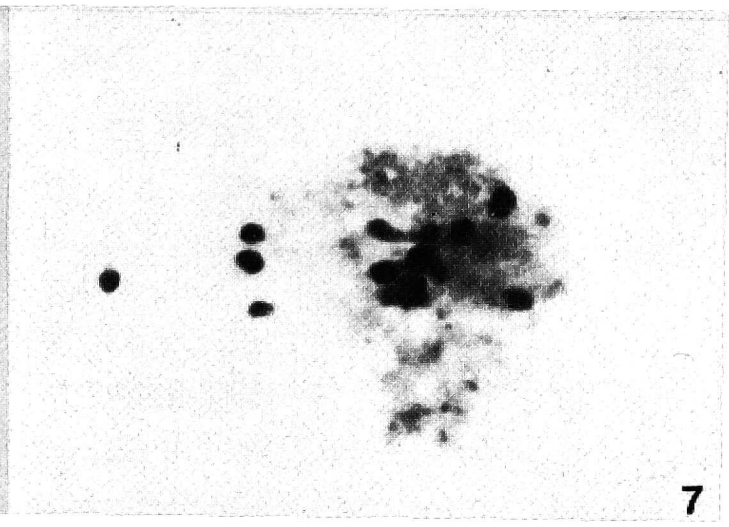
Table 2. The number of shoots and heads per plant, flowers per head and the shoot length in the parental species *T. pratense* and *T. diffusum*, and in their F_1 hybrids

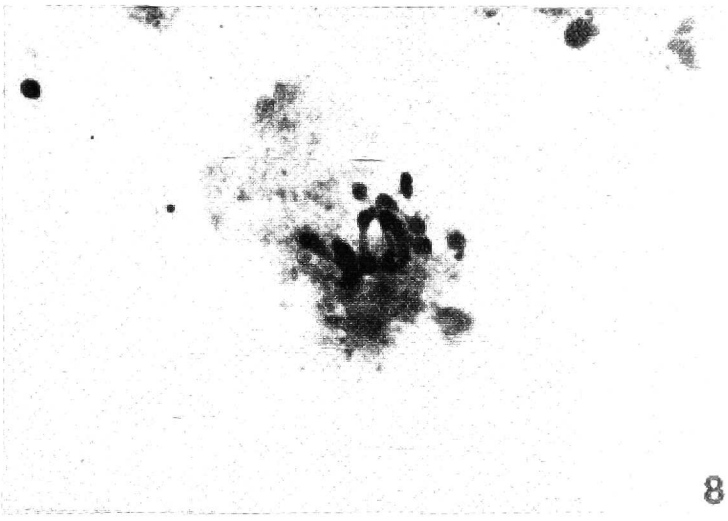
Genotype	Shoot length (cm)	No. of shoots	No. of heads	No. of flowers per head
T. pratense[1] (2n=16)	22.0	1.9	4.9	71.9
T. diffusum (2n=16)	23.6	1.0	6.0	42.0
F_1 (2n=16)	23.4	1.0	8.3	55.5

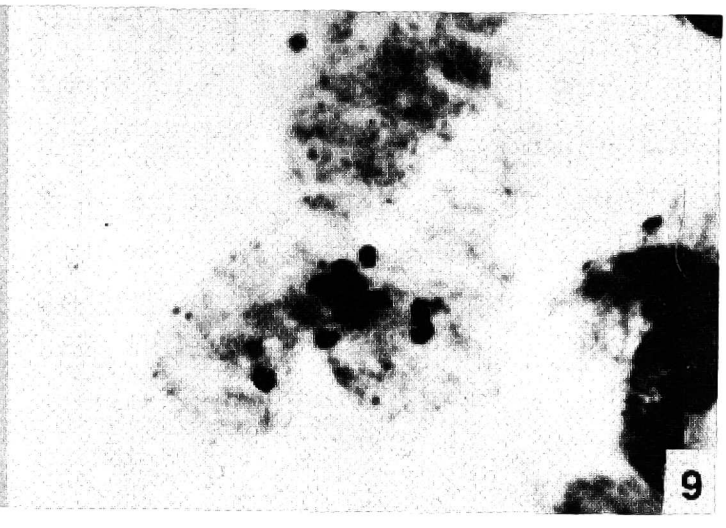
[1] *T. pratense* – a spontaneous form with 2n=14+2.

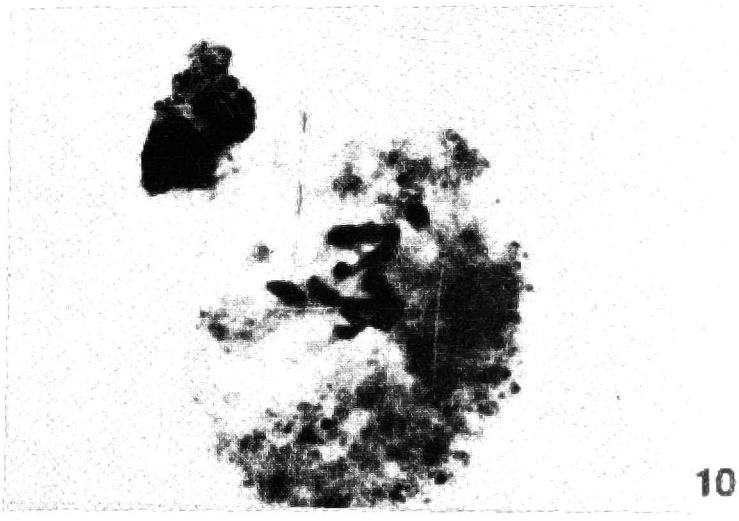


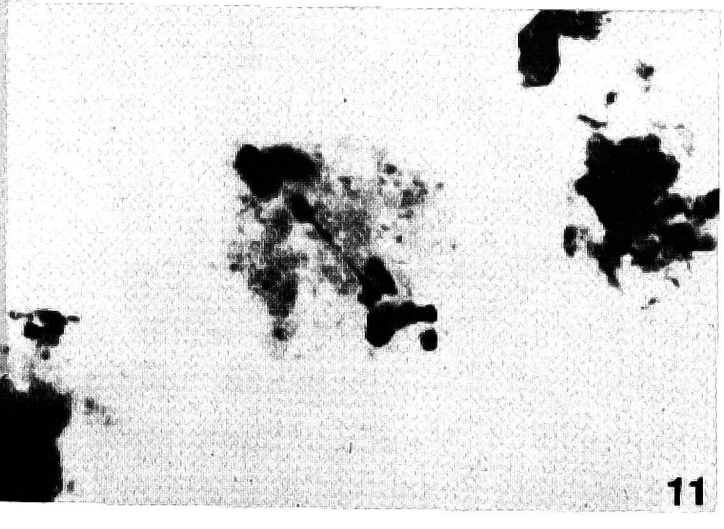


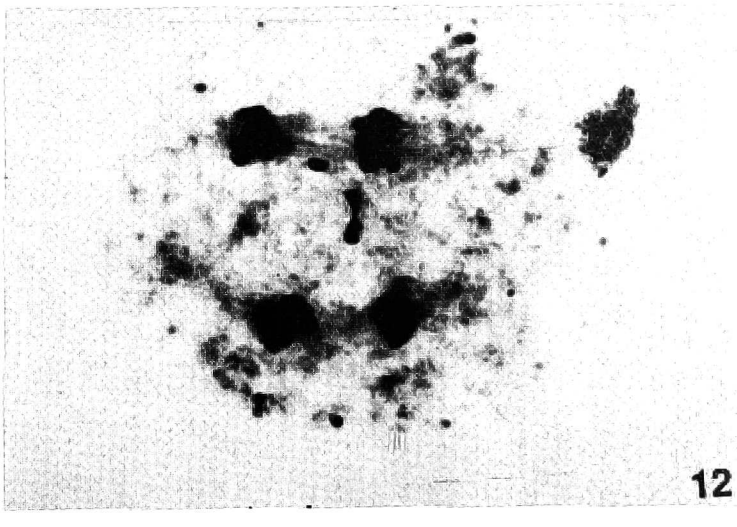












Figs. 5-12. Cell division in the F_1 hybrids *T. pratense* (2n=14+2) × *T. diffusum* (2n=16) somatic metaphase with 2n=16

Seeds collected after pollination of red clover (*T. pratense* 2n=16) with pollen of *T. apertum* (2n=16), *T. alexandrinum* (2n=16), *T. vavilovii* (2n=16), *T. hybridum* (2n=16), *T. hybridum* ssp. *anatolicum* (2n=16) did not give rise to hybrid plants (Table 1).

Table 3. The length (mm) of the flower and flower parts and the vexillum width (mm) in parental species *T. pratense* and *T. diffusum*, and in their F_1 hybrids

Genotype	Flower length	Flower tube length	Style length	Calyx sepals		Vexillum width
				shortest	longest	
T. pratense[1] (2n=16)	14.1	8.3	10.1	1.2	3.2	2.4
T. diffusum (2n=16)	10.4	6.5	7.4	4.8	7.3	1.5
F_1 (2n=16)	14.9	9.4	11.0	3.1	6.0	2.0

[1]*T. pratense* – a spontaneous form with 2n=14+2.

In meiosis during diakinesis and metaphase I in the hybrids (*T. pratense* 2n=16 × *T. diffusum* 2n=16), the chromosomes occurred in the form of bi-, tri and univalents. Only a single trivalent was observed in nine out of 146 analysed PMCs. The number of bivalents ranged from one to seven, that of univalents from 2 to 16. During metaphase I the following chromosome configurations were the most frequent: $3_{II}10_I$ (48.6%), $4_{II}8_I$ (10.2%) and 16_I (10.2%) (Figs. 6-8, Table 4). Chromosomes showed their high stickiness in numerous PMCs

Table 4. Metaphase I in 16-chromosome F_1 hybrids of *T. pratense* (2n=14+2) × *T. diffusum* (2n=16)

Chromosome patterns									No. of analysed PMCs
$7_{II}2_I$	$6_{II}4_I$	$5_{II}6_I$	$4_{II}8_I$	$3_{II}10_I$	$2_{II}12_I$	$1_{II}14_I$	$1_{III}3_{II}7_I$	16_I	
3	12	3	15	71	9	9	9	15	146

(about 30%) during that division stage. They formed compact conglomerations with several visible univalents in the equatorial plate (Fig. 9) or "end-to-end" branched chains (Fig. 10) consisting of 15-16 chromosomes. Bivalents of the hybrids were rod-shaped.

During anaphase I different chromosome numbers were seen on the opposite poles, whereas from 1 to 10 and even 11 lagging chromosomes resided in the

middle part of the cell. There occurred also chromosome bridges (Fig. 11). Pollen mother cells without lagging chromosomes, i.e. 10-6 and 9-7 chromosomes at each of the poles, constituted 62.4%. Also during anaphase II the chromosomes separated irregulary towards the progeny nuclei. Most cells (78.1%) were found to have from 1 to 10 chromosomes (Fig. 12) and sporadic chromosome bridges. Pollen mother cells after the second division transformed into tetrads and different from tetrads microspore polyads differing from one another in size. The most numerous were pentads – 58.4% and hexads – 22.6%. Tetrads constituted 10.5%, septads – 6.5%, octads – 1.5%. Nonads were less numerous – 0.2%. Mature pollen grains were inviable.

Discussion

Different methods have been developed by investigators to obtain interspecific hybrids within the genus *Trifolium*. Obtaining mature hybrids with *T. pratense* on a diploid level cultured both in vivo and in artificial media is not easy (TAYLOR et al. 1963, SCHWER, CLEVELAND 1972).

Difficulties with interspecific hybridization of *Trifolium* species, among others, due to inhibition of incompatible pollen tube growth in the pistil style were reported by KENDALL and TAYLOR (1965), WOJCIECHOWSKA (1989), ŚNIEŻKO and WINIARCZYK (1993).

Previous studies showed that two accessory chromosomes in the red clover genome probably reduce cross incompatibility (STRZYŻEWSKA 1984). On stabilization of a 16-chromosome form of *T. pratense* an attempt was made to cross it with other 16-chromosome *Trifolium* species. A previous analysis of chromosomes in the somatic cells showed that there is a certain differentiation in the chromosome size within the genotype of each species as well as between the species. All these species have a single pair of chromosomes with satellites (KAZIMIERSKI et al. 1972). It, however, appeared that after crossing *T. pratense* (2n=16) with *T. apertum*, *T. alexandrinum*, *T. vavilovii*, *T. hybridum*, *T. hybridum* ssp. *anatolicum* no hybrid plants were obtained likewise after crossing *T. diffusum* (female) to *T. pratense* (male) (2n=16). When *T. pratense* was a maternal form and *T. diffusum* – a paternal form, seeds and hybrid plants were successfully obtained. In the F_1 hybrid, traits of *T. diffusum* were dominant. That domination was found in the plant habit, in the shape of leaf blade, in the length of hairs on calyx sepals.

F_1 hybrids set no seeds in self- and cross-pollination. An analysis of pollen in Belling's agent showed that hybrid plants had no viable pollen. No seeds,

either, were obtained after pollination of hybrids with pollen of parental forms. This indicates that female gametes were also sterile.

The course of meiosis is not easy to interpret due to occurrence of various chromosome configurations and stickiness. At diakinesis and metaphase I the average number of bivalents per cell in 16-chromosome hybrids was 3.32 ranging from 1 to 7, the average number of univalents – 9.98 ranging from 2 to 16. The number of trivalents per cell ranged from 0 to 1. A low average number of bivalents as well as the occurrence of univalents during metaphase I indicate the lack of chromosome homology caused by structural changes. Bridges at anaphase I are an effect of crossing-over; they also prove the existence of structural heterozygosity.

The effect of irregularities during the first and second division were: unequal chromosome separation towards the poles, the loss of part of chromatin material as well as the formation of tetrads, pentads, hexads, septads, octads and nonads of microspores, which lost plasma in the further development and were inviable.

An analysis of chromosome conjugation showed that sterility of the F_1 hybrid *T. pratense* (2n=14+2) × *T. diffusum* (2n=16) is determined by the lack of homology of the chromosomes causing the death of reproduction male and female cells.

No hybrid plants have been obtained from the seeds originating from the remaining cross combinations. This, therefore, suggests that it is impossible to obtain hybrids of stable 16-chromosome *T. pratense* crossing it by the conventional method clover species with n=8 chromosomes (*T. apertum*, *T. alexandrinum* and others). It would be desirable to use in vitro cultures in studying hybrid embryos.

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