

## Amphidiploid hybrids of *Trifolium pratense* L. ( $2n=14+2$ ) with *T. diffusum* Ehrh. ( $2n=16$ )

Czesława STRZYŻEWSKA

Institute of Plant Genetics, Polish Academy of Sciences, Poznań

**Abstract.** After duplicating the chromosome number by colchicine in sterile  $F_1$  16-chromosome hybrid *T. pratense*  $\times$  *T. diffusum* some partially fertile plants with 32 chromosomes were found. Male fertility (viability of pollen grain) was from 69.3% to 86.2% (on average 81.8%), whereas female fertility estimated as seed setting after cross- and self-pollination was 21.8% and 6.9%, respectively. Male and female fertility as well as somatic chromosome number were examined in the  $F_2$ - $F_4$  generations. Selection for female fertility resulted in increasing seed setting in the first two generations ( $F_2$  and  $F_3$ ) and in decreasing it in  $F_4$  generation. An average seed setting in the  $F_2$ - $F_4$  generations after cross-pollination amounted to 22.2%, 43.6% and 12.9%, respectively; after self-pollination it was 25.2%, 27.6% and 1.9%. In the  $F_2$  generation all the plants had  $2n=32$  chromosomes. In the next generations there appeared aneuploids, among which 30-chromosome individuals were predominant.

**Key words:** amphidiploids, aneuploids, interspecific hybrids, *Trifolium diffusum*, *Trifolium pratense*

Interspecific crossing is one of the ways permitting to extend genetic variation in plant breeding when certain useful traits are transferred from wild to cultivated species. Investigations on obtaining interspecific hybrids from the genus *Trifolium* have been conducted for 60 years in many research centres. The main objective is to produce synthetic amphidiploid fertile and meiotically stable forms. Hybrids derived from crossing *T. pratense* L. ( $2n=14$ ) with *T. diffusum* Ehrh. ( $2n=16$ ) are male-sterile, though they may show a low female fertility in backcrosses with parental species (SCHWER, CLEVELAND 1972a). Hybrids of *T. pratense* ( $2n=14+2$ ) with *T. diffusum* ( $2n=16$ ) are completely

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Correspondence: Cz. STRZYŻEWSKA, Institute of Plant Genetics, Polish Academy of Sciences, Strzeszyńska 34, 60-479 Poznań, Poland.

male- and female-sterile (STRZYŻEWSKA 1995). The sterility barrier is usually overcome by crossing species having a duplicated chromosome number or through duplication of the chromosome number in  $F_1$  sterile hybrids.

Stabilized 16-chromosome forms of *T. pratense* with 16-chromosome *T. diffusum* give sterile  $F_1$  hybrids due to incomplete chromosome pairing in meiosis – their low homology (STRZYŻEWSKA 1995). In view of that, an attempt has been made to duplicate the chromosome number in that hybrid.

Results presented in this paper concern analysis of somatic chromosome number, morphological traits as well as male and female fertility in the  $F_1$ - $F_4$  generations of a 32-chromosome hybrid *T. pratense* ( $2n=14+2$ )  $\times$  *T. diffusum* ( $2n=16$ ).

## Material and methods

Sterile 16-chromosome  $F_1$  hybrids *Trifolium pratense* ( $2n=14+2$ )  $\times$  *T. diffusum* ( $2n=16$ ) (STRZYŻEWSKA 1995) were treated with 0.2% colchicine solution at the seedling stage to double the chromosome number. After the treatment, amphidiploid plants with  $2n=32$  chromosomes were obtained.

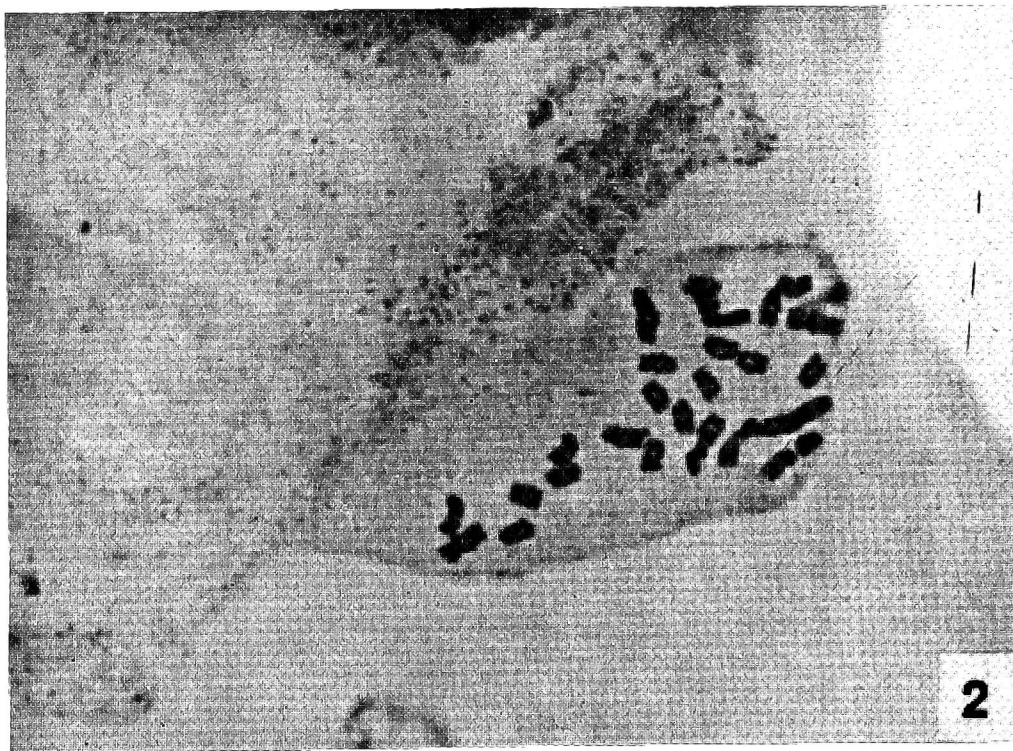
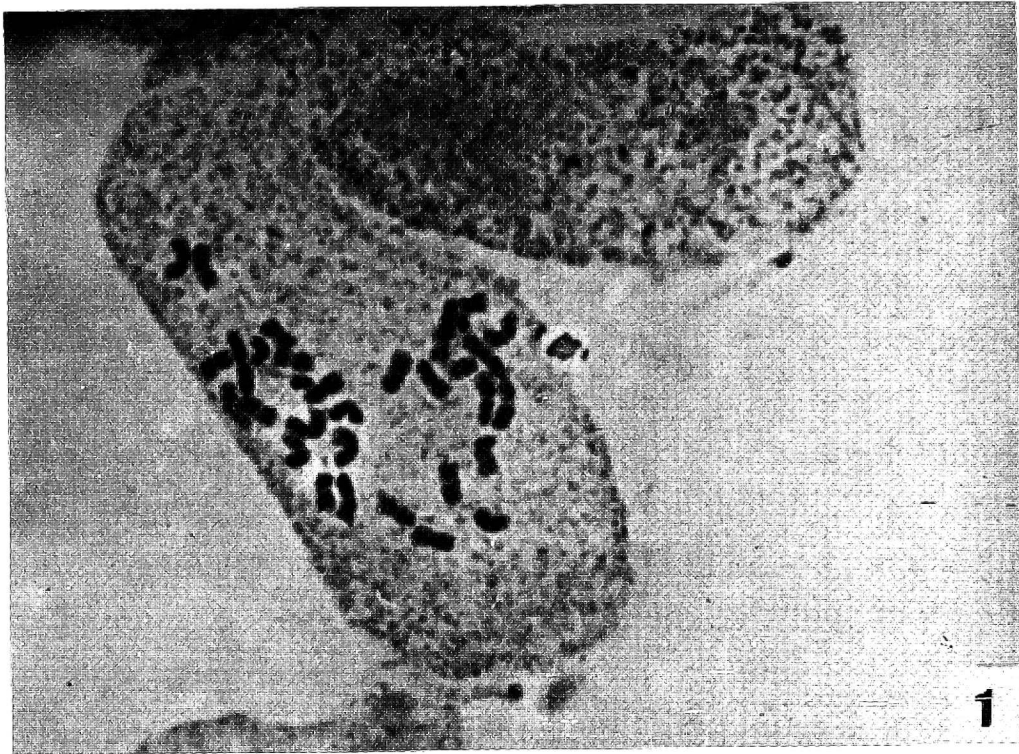
Fertility of amphidiploids in  $F_1$ - $F_4$  generations was evaluated after cross-pollination and after artificial self-pollination. In each generation plants with the highest seed setting were selected.

Pollen grain viability was estimated on the basis of the degree of their stainability in Belling's solution. A somatic chromosome number was examined in the root tip cells, which were fixed and stained on squashes according to the generally accepted methods (STRZYŻEWSKA 1995). Measurements of some morphological characters were carried out with particular reference to shoots length, number of shoots, heads and flowers per head as well as to flower structure.

## Results

On duplication of the chromosome number in 16-chromosome  $F_1$  hybrids, three out of 36 plants with  $2n=32$  chromosomes were separated. The obtained amphidiploid plants contained complete diploid chromosome complements of the parents, i.e. *T. pratense* and *T. diffusum*.

Plants with 32 chromosomes were less vigorous than their parental forms *T. pratense* and *T. diffusum* and resembled  $F_1$  hybrids with  $2n=16$  in the habit and some morphological traits (Table 3). They had one poorly leaved and branched stem without a rosette of 29.6 cm average length, with one, five and



**Figs. 1-2. Mitotic metaphase**  
1 – in amphidiploid plants with  $2n=32$ ; 2 – in aneuploid plants  
with  $2n=30$  chromosomes

**Table 1.** Average length and breadth (in mm) of some flower parts of *T. pratense* and *T. diffusum*, their F<sub>1</sub> hybrid and amphidiploids

Genotype	Chromosome number	Length of flower	Length of flower tube	Vexillum breadth	Pistil length
<i>T. pratense</i>	16	14.4	8.9	2.2	10.2
<i>T. diffusum</i>	16	10.4	6.5	1.5	7.4
F <sub>1</sub> <i>T. pratense</i> × <i>T. diffusum</i>	16	14.9	9.4	2.0	11.0
Amphidiploid plants*	32	16.1	8.9	2.0	11.6

\* after colchicine treatment of F<sub>1</sub> hybrid *T. pratense* (2n=14+2) × *T. diffusum* (2n=16).

seven heads, and with on average 40.8 flowers per head. Hybrids with 16 chromosomes had averagely 12.8 heads per shoot and 56.7 flowers per head. Amphidiploids had on the average slightly longer flowers and pistils, as compared to the parental forms and 16-chromosome hybrids, as well as a shorter flower tube. No differences were found in the vexillum breadth (Table 1).

Pollen viability of amphidiploids ranged from 69.3% to 86.2%, and the average diameter of pollen grains was 88.0 μ; in stable 16-chromosome *T. pratense* and *T. diffusum* it was 68.9 μ and 66.8 μ, respectively.

Amphidiploid F<sub>1</sub> plants in general were fertile. Seed setting after self-pollination ranged from 0.0% to 31.1% (with the average of 6.9%) and after cross-pollination it was on the average 21.8%. Seeds were well developed (Table 4).

**Table 2.** Somatic chromosome number in F<sub>2</sub>- F<sub>4</sub> generations of amphidiploids *T. pratense* × *T. diffusum*

Generation	No. of analysed plants	Plants with chromosome number (2n)				
		24	29	30	31	32
F <sub>2</sub>	27	—	—	—	—	27
F <sub>3</sub>	100	1	25	67	2	5
F <sub>4</sub>	248	—	19	218	9	2

The chromosome number of F<sub>2</sub>-F<sub>4</sub> plants are presented in Table 2. In the F<sub>2</sub> generation all plants had 2n=32. Five plants in F<sub>3</sub> and only two plants in F<sub>4</sub> were found to have the chromosome number 2n=32 (16+16). The remaining plants had an aneuploid chromosome number from 2n=24 to 2n=31, 30-chromosome plants being predominant (Figs. 1, 2).

In  $F_2$  generation 31 seedlings were observed to be green and one was albinotic (it died at the stage of several leaves). In the  $F_3$  generation, 17 out of 117 seedlings were albinotic, while the remaining plants had yellow leaves with large or small chlorophyll spots. In mature plants chlorophyll coloration occurred on rosette and shoot leaves as well as on calyx sepals. In  $F_2$  and  $F_3$  plants a large variation was noticed with regard to some morphological traits (including stem length and head number) as well as vigour and earliness.

Amphidiploids in  $F_2$  generation were similar to  $F_1$  plants ( $2n=32$ ) in its habit. Hybrid plants of  $F_2$  were characterized by an erect type of growth and

**Table 3.** The length of shoots and the number of shoots, heads and flowers per head in amphidiploids *T. pratense*  $\times$  *T. diffusum*

Genera- tion	Shoot length (cm)		Number of shoots		Number of heads		Number of flowers per head	
	range	$\bar{x}$	range	$\bar{x}$	range	$\bar{x}$	range	$\bar{x}$
$F_1$	20.0-43.0	29.6	1	1	1-7	4.3	20-63	40.8
$F_2$	21.0-56.0	38.2	1-3	1.9	3-25	13.7	30-61	42.3



**Fig. 3.** Seedlings in amphidiploid ( $2n=32$ ) and aneuploid ( $2n=30$ ) plants

**Table 4.** Seed setting and pollen viability in the F<sub>1</sub>-F<sub>4</sub> generations of *T. pratense* x *T. diffusum*

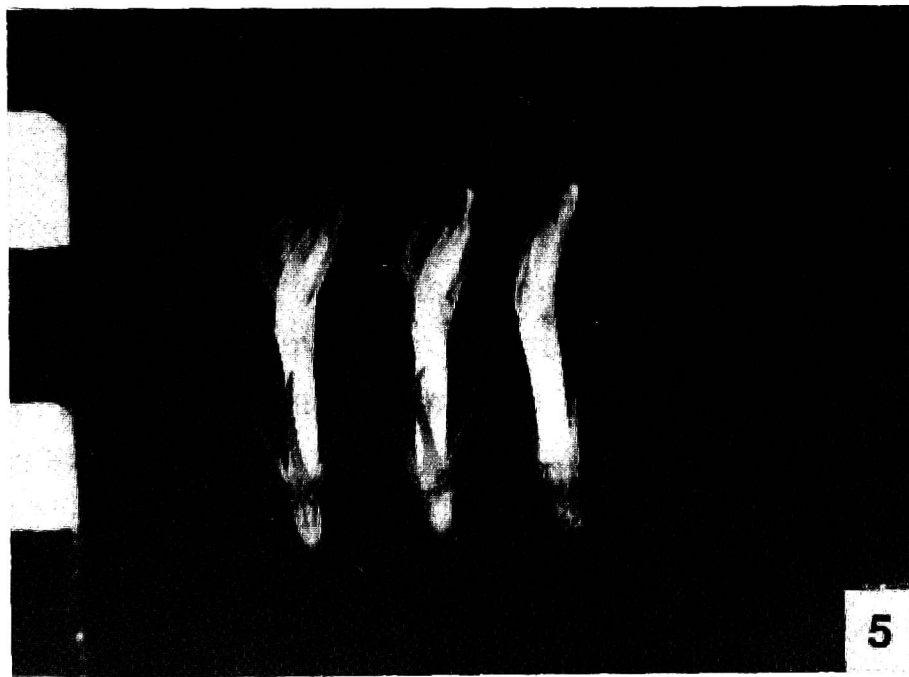
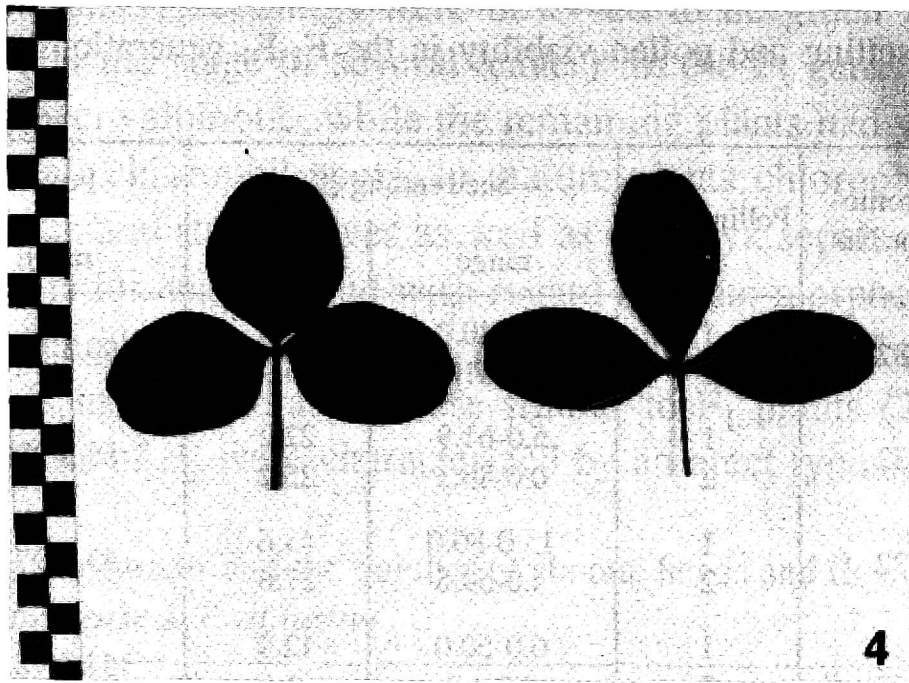
Generation	Chromosome No.	Pollination*	Seed setting (%)		Pollen viability (%)	
			range	$\bar{x}$	range	$\bar{x}$
F <sub>1</sub>	32	1	0.0-40.0	21.8	69.3-86.2	81.8
		2	0.0-31.0	6.9		
F <sub>2</sub>	32	1	5.0-61.9	22.2	85.3-90.8	88.7
		2	0.0-86.3	25.2		
F <sub>3</sub>	32	1	15.0-80.0	43.6	83.1-97.5	93.0
		2	4.0-59.6	27.6		
F <sub>4</sub>	32	1	0.0-50.0	12.9	54.7-78.3	66.5
		2	0.0-4.3	1.9		
	30	1	5.0-80.0	35.2	77.3-82.0	79.4
2	0.0-50.0	23.0				

\* 1 – cross-pollination, 2 – self-pollination

by a small leaf rosette. Shoots were few (on average 1.9 per plant) and poorly branched and leaved, with the average length of 38.2 cm. The shoots had from 3 to 25 heads with the average number of flowers 42.3 (Table 3). They came to flower two weeks earlier than 16-chromosome plants of *T. pratense*.

Pollen viability and seed setting in F<sub>2</sub>-F<sub>4</sub> generation are shown in Table 4. No large variation in pollen viability was found and its average value was high amounting to 88.7% in the F<sub>2</sub> generation and 93.0% in the F<sub>3</sub>. Large variation was found in seed setting in both F<sub>2</sub> and F<sub>3</sub> generations. But the average degree of seeds setting at both cross- and self-pollination was higher in the F<sub>3</sub> generation (43.6% and 27.6%, respectively) than in the F<sub>2</sub> (22.2% and 25.2%, respectively). Amphidiploid plants had (on average) 66.5% of stainable pollen grains in the F<sub>4</sub> generation. The degree of seed setting after self- and cross-pollination was markedly lower than that in the F<sub>1</sub> - F<sub>3</sub> and amounted to 1.9% and 12.9%, respectively.

In the F<sub>4</sub> generation, besides plants with the chromosome number 2n=32, individuals with 2n=30 have segregated. Seedlings of these individuals showed chlorophyll defects. They differed from amphidiploid plants by the leaf blade index and some morphological characters of flowers such as the length of a flower tube and the longest calyx sepal, setaceous erect hairs on the calyx and its sepals (Figs. 3-6). In vigour they were similar to 32-chromosome plants, but set seeds better. The average pollen viability was high (79.4%) and ranged from 77.3% to 82.0%. The variation range with regard to the degree of seed setting was wide – from 5.0% to 80.0% at cross-pollination and from 0.0% to



Figs. 4-6. Flowers and leaves in amphidiploid ( $2n=32$ ) and aneuploid ( $2n=30$ ) plants  
4 – leaves, on the left – amphidiploid, on the right – aneuploid , 5 – flowers in amphidiploid  
plants with  $2n=32$ , 6 – flowers in aneuploid plants with  $2n=30$ ,

50,0% at self-pollination (Table 4). The remaining aneuploids were less viable and had a decreased fertility.

## Discussion

By crossing tetraploid forms of *T. pratense* ( $2n=4x=28$ ) and *T. diffusum* ( $2n=4x=32$ ), TAYLOR et al. (1963), SCHWER and CLEVELAND (1972b), TAYLOR et al. (1973) obtained fertile hybrids. Known are also hybrids of *T. pratense* with other clover species derived from in vitro culture of immature embryos. MERKER (1988) obtained amphidiploids of *T. alpestre* and *T. pratense* after colchicine treatment. Only in one out of 8 hybrid plants the chromosome number was  $2n=30$  (16+14). This form appeared to be male-sterile.

In the presented studies as a result of colchicine treatment of sterile 16-chromosome  $F_1$  hybrids *T. pratense* ( $2n=14+2$ ) × *T. diffusum* ( $2n=16$ ) individuals with a doubled chromosome number  $2n=32$  were selected. The obtained plants in the  $F_1$ - $F_3$  generations exhibited a high male fertility and as a rule – a lower female fertility, though there was a large variation of that trait in some cases, i.e. plants distinguishing by a high degree of seed setting occurred beside completely sterile plants. Data presented in this paper show that after selection for fertility increase in females, their average fertility in the  $F_1$ - $F_3$  generations gradually increased as a result of both cross- and self-pollination. In the  $F_4$  generation amphidiploids were found to have a decrease in their male and female fertility. Pollen viability declined by about 27%, and seed setting was by almost 30% lower than that in  $F_3$ .

As reported by SCHWER and CLEVELAND (1972b) in the studies concerning the first two generations of the cross *T. pratense* × *T. diffusum*, seed setting in allopolyploid forms was satisfactory and amounted to about 7% in  $F_1$  and to 33% – in  $F_2$ . Similar hybrid plants but with a low degree of seed setting (1%) after self-pollination were obtained by TAYLOR et al. (1973). POZDNYAKOV (1991) derived tetraploid plants *T. pratense* ( $2n=4x=28$ ) × *T. diffusum* ( $2n=4x=32$ ), which set seeds to 37.9 + 13.4% in the  $F_1$ - $F_2$  generations.

The most important in studies on interspecific hybrids is obtaining cytogenetically stable and fertile forms. A low cytogenetic stability in amphidiploids is a result of irregularities in the meiosis, which as a consequence leads to aneuploidy. The occurrence of aneuploids in populations of induced tetraploids and their hybrids is well-known. In the present studies, all  $F_2$  plants had 32 chromosomes in their somatic cells. In the next generations of amphidiploids there appeared aneuploid individuals. These were hypoploids ( $2n<32$ ), among which plants with  $2n=30$  were the most numerous. Aneuploid with  $2n=30$  set



seeds better and had more viable pollen than 32-chromosome individuals of the  $F_4$  generation. The remaining aneuploid, however, were less viable and had a decreased fertility. A low cytogenetic stability is a significant reason of a decreased fertility in amphidiploid plants *T. pratense* ( $2n=14+2$ )  $\times$  *T. diffusum* ( $2n=16$ ) in the  $F_4$  generation. A significant portion of aneuploid individuals in the  $F_3$  and  $F_4$  generation was a result of irregularities in microsporogenesis. This is also indicated by a decreased pollen viability of amphidiploid plants in the  $F_4$  generation in comparison to 32-chromosome hybrids of the  $F_3$  generation.

In the  $F_4$  generation only two plants had the chromosome number  $2n=32$  ( $16+16$ ). Further studies will permit to elucidate whether observed crisis of stability and fertility concerns only this generation and whether the genome of amphidiploid clover  $2n=32$  (*T. pratense*  $2n=14+2$  and *T. diffusum*  $2n=16$ ) will be little stable and 30-chromosome plants will persist in this hybrid populations. Aneuploid individuals with  $2n=30$  chromosomes set seeds better and had more viable pollen than plants with  $2n=32$  ( $16+16$ ) chromosomes.

The data presented in this paper show that the genome of amphidiploid *T. pratense* ( $2n=14+2$ )  $\times$  *T. diffusum* ( $2n=16$ ) became stable at the level of  $2n=30$  and not, as it was assumed, at the level of  $2n=32$  chromosomes. It may be that two of additional chromosomes in *T. pratense* ( $2n=14+2$ ) or two others, the absence of which causes no decrease in the fertility of 30-chromosome hybrid plants, are eliminated.

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