

## Investigations on hybrids in the genus *Trifolium* L.

### V. Fertility and cytogenetics of the hybrid *Trifolium nigrescences* Viv. × *T. isthomocarpum* Brot.

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#### Abstract

*T. nigrescens* ( $2n = 16$ ) crosses with *T. isthomocarpum* ( $2n = 16$ ) reciprocally. The viability of hybrid seedlings depend from the direction of the cross. At the time of diakinesis and metaphase I the average chromosome figures per PMC's was 7.014<sub>II</sub>, 0.005<sub>III</sub>, 0.435<sub>IV</sub> and 0.209<sub>I</sub>. For one half of the PMC's in the metaphase I the typical chromosome arrangement was 8<sub>II</sub>. The F<sub>1</sub> plants was almost completely sterile. The causes of viability of hybrid seedlings depending on the direction of the cross, and the sterility of hybrid plants, are discussed.

#### INTRODUCTION

The interspecific hybrids of *Trifolium* with  $2n = 16$  chromosomes known to date exhibit normal fertility (Kazimierski and Kazimierska, 1970, 1972; Kazimierski, Kazimierska and Strzyżewska, 1972). The hybrids here described developed from the cross *Trifolium nigrescens* and *T. isthomocarpum*, species with also  $2n = 16$  chromosomes. Both these species cross with *T. repens* ( $2n = 32$ ) giving but little fertile or sterile hybrids. Since *T. nigrescens* and *T. isthomocarpum* cross with *T. repens* although having one half the number of chromosomes of the latter, they are considered as the probable ancestors of white clover which is believed to be a natural tetraploid (Brewbaker and Keim, 1953; Evans, 1962; Kazimierski and Kazimierska, 1970, 1972). Investigation of interspecific hybrids considered as the possible components in the development of the tetraploid species *T. repens*, at the level of natural ploidy,

and later in induced allopolyploids, may elucidate the relationship between the chromosomes of the genomes of the species crossed, and further the behaviour of these genomes in an amphidiploid organism. The present paper gives a characteristic of some traits and properties of a hybrid at the diploid level.

#### MATERIAL AND METHODS

The *T. isthomocarpum* Brot. seeds were received from Israel. This species is an annual plant. The dark green leaves appear in two shapes, in the lower part of the stem they are obovate, and in the upper their shape is close to rhomboid. The plants are not high, 25 cm on the average. The flowers are large, heather-pink, set on the widened apical part of the inflorescence stalk. After overblowing the flowers do not droop, the perianth turns brown but does not fall off. The plants flower profusely, on the average there are 60 flowers in a head. The stems are green with anthocyanin strands; particularly much of this substance is accumulated in the stem nodes. The ovary contains two ovules, the pod has one or two purple seeds, thousand seed weight is 0.52 g.

The seeds of *T. nigrescens* Viv. originated from the collection in Gatersleben (German Democratic Republic). This species is also an annual plant. The leaves are small, green, almost square. The flowers are small, white, set at the end of the inflorescence stalk on peduncles, after the end of flowering they drop. The perianth does not fall off, the ripe heads are dark brown. The plants flower profusely, there are on the average 22 flowers in a head. The stems are thin, delicate, green, the internodes are short with anthocyanin strands. The ovary contains four ovules and the pod 1—4 yellow minute seeds, thousand seed weight is 0.27 g.

The opening flowers were emasculated and pollinated on the same day with freshly collected pollen from plants of the paternal species.

The parental and  $F_1$  forms grew in a well isolated glass-house, and when part of the  $F_1$  plants came into bloom they were transferred out of doors to see how they would set seeds after pollination by insects.

The fertility of the female gametes was checked by crossing the  $F_1$  plants with one another and by back-crossing with plants of the parent species. Microspore fertility was determined by staining the pollen grains with Belling's reagent.

For cytological analysis small flower buds were fixed in Carnoy solution and preserved in 70 per cent alcohol. The course of meiosis and microsporogenesis was investigated on smears stained with acetocarmine.

## RESULTS

The plants of the parent species are self-incompatible. Crossed between themselves *T. isthomocarpum* and *T. nigrescens* set on the average 1.63 and 3.13 seeds, respectively, per pollinated flower (Table 1). Seeds of hybrids between *T. nigrescens* and *T. isthomocarpum* were obtained from crosses in both directions. The mean number of ripe seeds per pollinated flower in crosses of the two above mentioned species was lower than in cross pollination within one species (Table 1).

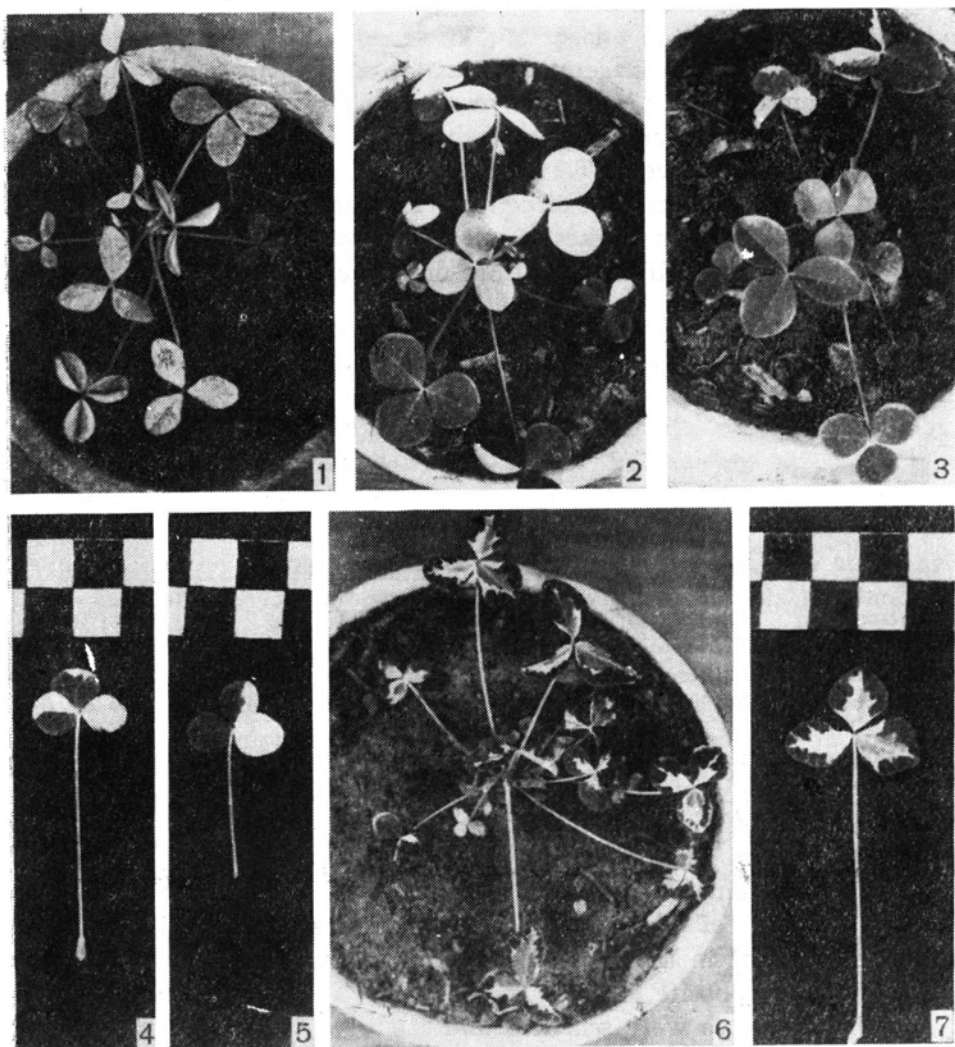
Table 1  
Crossing, seed setting and viability of seedlings

Species		Number of		Mean no. of seeds per pollinated flower	Seedlings		Flowering plants	
♀	♂	pollinated flowers	ripe seeds		no.	%	no.	%
<i>T. nigrescens</i>	<i>T. nigrescens</i>	60	188	3.13				
<i>T. isthomocarpum</i>	<i>T. isthomocarpum</i>	100	163	1.63				
<i>T. nigrescens</i>	<i>T. isthomocarpum</i>	175	261	1.49	158	60.5	33	20.9
<i>T. isthomocarpum</i>	<i>T. nigrescens</i>	106	58	0.54	38	65.5	33	86.8

Analysis of the number of ovules in the ovary showed that *T. isthomocarpum* plants have on the average two, and *T. nigrescens* plants four ovules. Thus, the per cent of ripe seeds per pod in crosses between *T. isthomocarpum* plants is 80.1 per cent and between *T. nigrescens* plants 78.3 per cent. Calculation of the per cent of seeds set in relation to the number of ovules in the ovary for interspecific crosses demonstrated that in the cross *T. isthomocarpum* × *T. nigrescens* it is 27.3 and in the opposite cross 37.2%.

The hybrid seeds were scarified and seeded on moist filter paper on Petri dishes. After several days the germinating seeds were transferred to pots filled with soil and peat. On the average 60 per cent of the seeds germinated. The seeds from the cross *T. isthomocarpum* × *T. nigrescens* germinated somewhat better than those from the opposite cross (Table 1).

The direction of crossing became manifest during farther growth of the plants. From the cross *T. isthomocarpum* × *T. nigrescens* almost 87 per cent of plants came into flower, and from the opposite cross less than 21 per cent (Table 1). The lower viability of the cross *T. nigrescens* × *T. isthomocarpum* than of the other one was due to the fact that most seedlings of the *T. nigrescens* × *T. isthomocarpum* cross were albinotic, others had only small irregular green spots on the cotyledons. Such seedlings were unable to synthesize chlorophyll and died within two or three weeks after sowing. Other seedling which had on the white



Photos 1—7. Plants and leaves of  $F_1$  *T. nigrescens*  $\times$  *T. isthmocarpum*

1 — Plant with variegated marbled leaves, on the white background of leaves blade a small green spot; 2 and 3 — Sector chimaera plants; 4 and 5 — Leaves from the chimaera sector of plants; 6 and 7 — Periclinal chimaera plant and leaf from such a plant

background of the cotyledones and leaf blades minute grey-green spots grew very slowly, developed several or more than ten leaves (Photo 1) and died before efflorescence. In the case when the seedling exhibited a chlorophyll-free and green segment, that is it was a segmental chlorophyll chimaera (Photos 2—5) or when only part of the palisade layer of the leaf blade was deprived of the ability of chlorophyll synthesis and the plant was a periclinal chimaera (Photos 6 and 7), the plants grew slower and flowered later. The segmental chimaerae preserved the chlorophyll-less segment to the end of the vegetation

period, so did the periclinal chimerae persist to the end as chimaerae.

Plants obtained from seeds of the reverse cross (*T. isthomocarpum* × *T. nigrescens*) showed no disturbances in chlorophyll production.

### Some morphological traits

The leaves of the parent species differed in shape and size (Tables 2 and 3). The shape and size of the leaves changes within the plant from below to the apex. In *T. isthomocarpum* the leaves both from the lowest and higher nodes were longer and wider than those of *T. nigrescens* and they had somewhat shorter petioles. The leaves of F<sub>1</sub> plant were more similar in size and shape to those of *T. nigrescens*; they were in general set on shorter petioles than in the parental forms.

Table 2

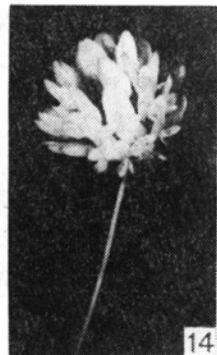
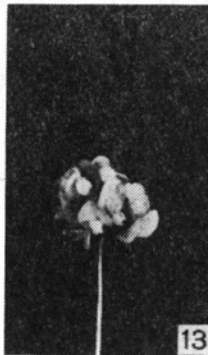
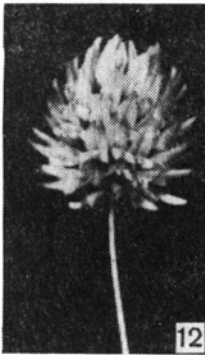
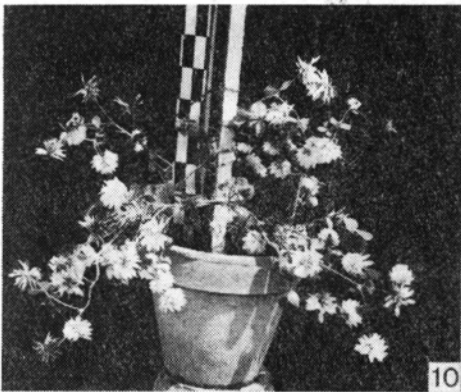
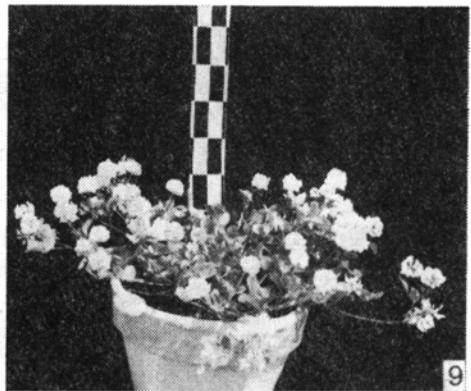
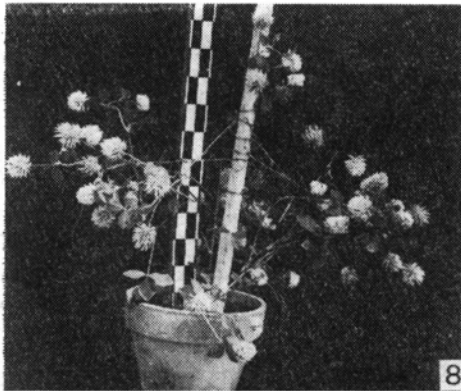
Mean length and width of leaves in whorls and leaves from stem in *T. isthomocarpum*, *T. nigrescens* and F<sub>1</sub> plants

Species	Leaves in whorl, mm		Length to width ratio in leaf blade	Leaves from stem, mm		Length to width ratio in leaf blade
	length	width		length	width	
<i>T. isthomocarpum</i>	16.5	12.2	1.35	19.2	12.6	1.53
<i>T. nigrescens</i>	12.3	10.9	1.13	11.7	9.4	1.24
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	11.6	10.4	1.11	12.0	8.8	1.37
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	12.5	10.8	1.15	13.7	10.5	1.25

Tabela 3

Length of petioles and inflorescence stalks in plants of parental species and F<sub>1</sub> plants

Species	Length of petiole (mm) of leaf from:						Length of inflorescence stalk (mm)		
	whorl			stem			mini- mum	maxi- mum	mean
	mini- mum	maxi- mum	mean	mini- mum	maxi- mum	mean			
<i>T. isthomocarpum</i>	3.2	7.8	4.95	0.3	1.8	0.70	2.2	5.5	3.48
<i>T. nigrescens</i>	3.3	7.2	5.02	0.4	2.1	1.07	1.5	5.0	2.81
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	2.7	6.1	4.10	0.3	1.0	0.50	1.8	5.1	2.92
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	2.0	6.8	4.26	0.2	2.0	1.00	1.9	5.1	2.91



Photos 8—14. Plants and inflorescences of parental forms and  $F_1$

8 — *T. isthomocarpum*; 9 — *T. nigrescens*; 10 —  $F_1$  *T. isthomocarpum*  $\times$  *T. nigrescens*; 11 —  $F_1$  *T. nigrescens*  $\times$  *T. isthomocarpum*; 12, 13, 14 — Inflorescences: *T. isthomocarpum* (12), *T. nigrescens* (13),  $F_1$  *T. isthomocarpum*  $\times$  *T. nigrescens* (14)

The general appearance of the  $F_1$  plants resembled rather *T. nigrescens* (Photo 8—11). The *T. isthomocarpum* plants were on the average higher than the *T. nigrescens* ones (Table 4);  $F_1$  plants of *T. isthomocarpum*  $\times$  *T. nigrescens* reached a somewhat greater length than *T. nigrescens* specimens and the  $F_1$  plants of *T. nigrescens*  $\times$  *T. isthomocarpum* were almost as high as *T. isthomocarpum* plants.

Table 4

Variability of height of parental and F<sub>1</sub> plants

Species	Height of plants, cm				Total no. of plants
	up 10	11—20	21—30	31—40	
<i>T. isthomocarpum</i>		4	8	4	16
<i>T. nigrescens</i>	1	7	2		10
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>		5	10		15
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>		4	4	3	11

Table 5

Variability of beginning of flowering of parental form and F<sub>1</sub> plants

Species	Dates of efflorescence				Total no. of plants
	1—10 June	11—20 June	21—30 June	1—10 July	
<i>T. isthomocarpum</i>	9	1			10
<i>T. nigrescens</i>		3	5	2	10
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	4	18	5	2	29
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	1	7	16	7	31

Table 6

Variability of number of florets in head of parental and F<sub>1</sub> plants

Species	No. of florets in head									Total no. of heads	Mean no. of flowers in head
	up to 15	16— 25	26— 35	36— 45	46— 55	56— 65	66— 75	76— 85	86— 95		
<i>T. isthomocarpum</i>			1	11	46	80	47	12	5	203	60.4
<i>T. nigrescens</i>	7	137	46							190	22.2
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>		58	121	21						200	28.1
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>		25	75	54	6	1				161	32.7

The first to bloom were the *T. isthomocarpum* plants and the earliest among the hybrids (Table 5). More than 46 per cent of F<sub>1</sub> plants of the cross *T. isthomocarpum* × *T. nigrescens* flowered ten days earlier than did about 51 per cent of F<sub>1</sub> plants of the opposite cross.

*T. isthomocarpum* has the longest inflorescence stalks, in *T. nigrescens* they are on the average by 0,7 cm shorter (Table 3). The F<sub>1</sub> plants had stalks shorter than those of *T. isthomocarpum* but somewhat longer than those of *T. nigrescens*.

Of the two species crossed more flowers are found in the heads of *T. isthomocarpum*, on the average 60 and in *T. nigrescens* 22 flowers (Table 6). The F<sub>1</sub> plants of the cross *T. isthomocarpum* × *T. nigrescens* had six, and those of the reverse cross 10 flowers more in the head than *T. nigrescens* plants (Photos 12—14).

Table 7

Size of flowers in plants of parental species and F<sub>1</sub> plants

Species	Length, mm						Width of standard mm			Length of calyx mm		
	of flower			of standard			mini- mum	maxi- mum	mean	mini- mum	maxi- mum	mean
	mini- mum	maxi- mum	mean	mini- mum	maxi- mum	mean						
<i>T. isthomocarpum</i>	10,0	12,4	11,0	6,4	7,9	6,9	2,0	3,0	2,6	4,3	5,0	4,7
<i>T. nigrescens</i>	6,9	8,2	7,7	4,0	5,9	4,7	3,2	4,0	3,8	2,4	5,6	3,6
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	9,4	11,9	10,6	6,2	7,3	6,9	2,4	3,5	2,7	4,1	5,0	4,6
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	7,3	8,7	8,2	5,1	6,0	5,7	2,1	3,1	2,6	4,0	4,9	4,6

The flowers are longer with an elongated calyx in *T. isthomocarpum* (Table 7). The standard in *T. nigrescens* is wider than in *T. isthomocarpum* flowers. Measurements on F<sub>1</sub> plants showed that their flowers are only slightly smaller or equal to those of *T. isthomocarpum*; the width of the standard was equal to that in *T. isthomocarpum*, thus it was narrower than in *T. nigrescens*.

The F<sub>1</sub> plants like the paternal ones had heather-pink flowers on pedicels 1.0—1.5 mm long at the somewhat thickened end of the inflorescence stalk. After the end of flowering the flower in the heads of F<sub>1</sub> plants drooped similarly as in *T. nigrescens*.

Measurements of the stomata on the lower side of the leaf blade demonstrated that the *T. nigrescens* and F<sub>1</sub> plants have longer and wider stomata than has *T. isthomocarpum* (Table 8). The number of stomata per surface area unit of the leaf of the F<sub>1</sub> plant was intermediate between those of the parental forms. Separate measurements on the green and chlorophyll-deprived segments of the chimaerae showed certain differences in the dimensions and number of stomata between the green and chlorophyll-deprived segments of segmented chimerae and between the green part of the leaf blade and the grey-green part in periclinal chimaerae (Table 8). If we take into account the variability



Table 8

Dimensions and number of stomata on lower surface of leaf in plants of parental species and F<sub>1</sub> plants

Species	Stomata (mean) μ		No. of stomata in field of vision of microscope (magnif. 10×44)			No. of plants examined
	length	width	mini- mum	maxi- mum	mean	
<i>T. isthomocarpum</i>	25.6	12.7	11	23	15.3	4
<i>T. nigrescens</i>	27.5	17.2	7	18	12.7	4
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	26.7	17.2	7	29	13.3	13
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	28.5	18.6	7	23	14.4	7
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i> (segmental chimera) leaves						
from green segment	25.4	17.0	14	24	19.1	1
from white segment	27.4	16.8	9	22	17.0	
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i> (periclinal chimera) from:						
green part of leaf	28.5	13.8	9	12	10.7	1
discoloured part of leaf	26.6	17.0	14	23	17.6	

of the above named traits in hybrids without chlorophyll disturbances, the deviations noted in the chlorophyll chimaerae do not exceed the limits of this variability.

#### Hybrid fertility

F<sub>1</sub> plants growing in the isolated glasshouse did not set seeds when crossed between themselves and pollinated by bees they gave sporadic single seeds. The flowers of the F<sub>1</sub> plants pollinated with pollen from the parental plants gave only few ripe seeds (Table 9). Plants of the parental species pollinated with F<sub>1</sub> pollen set seeds. The per cent of ripe seeds in relation to the number of ovules in the ovary in *T. nigrescens* × F<sub>1</sub> (*T. isthomocarpum* × *T. nigrescens*) was 28.8 and after pollination of *T. isthomocarpum* with F<sub>1</sub> pollen 66.6 per cent (Table 9).

Seeds collected from F<sub>1</sub> and parental plants pollinated with F<sub>1</sub> pollen when seeded on a germination apparatus swelled but seldom germinated. Of the total number of 89 seeds two plants developed, one of which from *T. nigrescens* pollination and the second from *T. isthomocarpum* pollination with F<sub>1</sub> pollen. The former plant with *T. nigrescens* as maternal form resembled *T. nigrescens*, the latter derived from *T. isthomocarpum* as maternal form was similar to the maternal plant, both proved to be sterile.

Table 9

Seed setting after selfpollination, cross pollination and back pollination by F<sub>1</sub> plants

Species		No. of	
		pollinated flowers	ripe seeds
♀	♂		
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	3329	10
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	13091	11
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	<i>T. isthomocarpum</i>	171	8
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	<i>T. nigrescens</i>	65	1
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	<i>T. isthomocarpum</i>	45	2
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	<i>T. nigrescens</i>	45	2
<i>T. nigrescens</i>	F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	13	15
<i>T. isthomocarpum</i>	F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	30	40
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	selfpollination	7529	0
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	selfpollination	8907	0

Table 10

Pollen fertility in *T. isthomocarpum*, *T. nigrescens* and F<sub>1</sub> plants

Species	Fertile pollen grains, %						No. of plants examined
	up to 10.0	10.1—20.0	.....	70.1—80.0	80.1—90.0	90.1—100.0	
<i>T. isthomocarpum</i>				2	3	5	10
<i>T. nigrescens</i>				1	2	7	10
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	16	13					29
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	17	14					31

The pollen fertility of the parental forms was high (Table 10) it varied within the limits of 70—100 per cent. Among the *T. isthomocarpum* plants one half and among the *T. nigrescens* ones 70 per cent of the individuals exhibited 90 per cent fertility. F<sub>1</sub> plants of both crosses showed a pollen fertility of 3.1—17.0 per cent; in both hybrids more than 50 per cent of the plants belonged to the group with fertility less than 10 per cent, the remaining had a higher fertility (Table 10).

Of the two species under study *T. isthomocarpum* has larger pollen grains with an average diameter of 31.9  $\mu$ , the average diameter of *T. nigrescens* pollen grains is 24.4  $\mu$ . F<sub>1</sub> plants of the *T. isthomocarpum* × *T. nigrescens* cross had pollen with an average diameter 27.7  $\mu$ , and those of the reverse cross with a 27.2  $\mu$  diameter. Thus, the pollen grains of the F<sub>1</sub> plants had a diameter intermediate between those of the parental forms.

Variability of the pollen grain diameter within the parental and  $F_1$  forms was high (Diagram 1). In the parental forms, however, there were but few grains of extreme dimensions, and on the diagram only the beginnings and ends of the curves overlap where the number of very large or very small grains was very low, and the peaks with the most frequent diameters lie at considerable distances from one another. The curves of frequency of the pollen grain diameters for  $F_1$  plants are very similar to one another, and their peaks occupy an intermediate position between the peaks of the curves for the parental forms. The per cent of pollen grains with extreme dimensions is small similarly as in the parental forms.

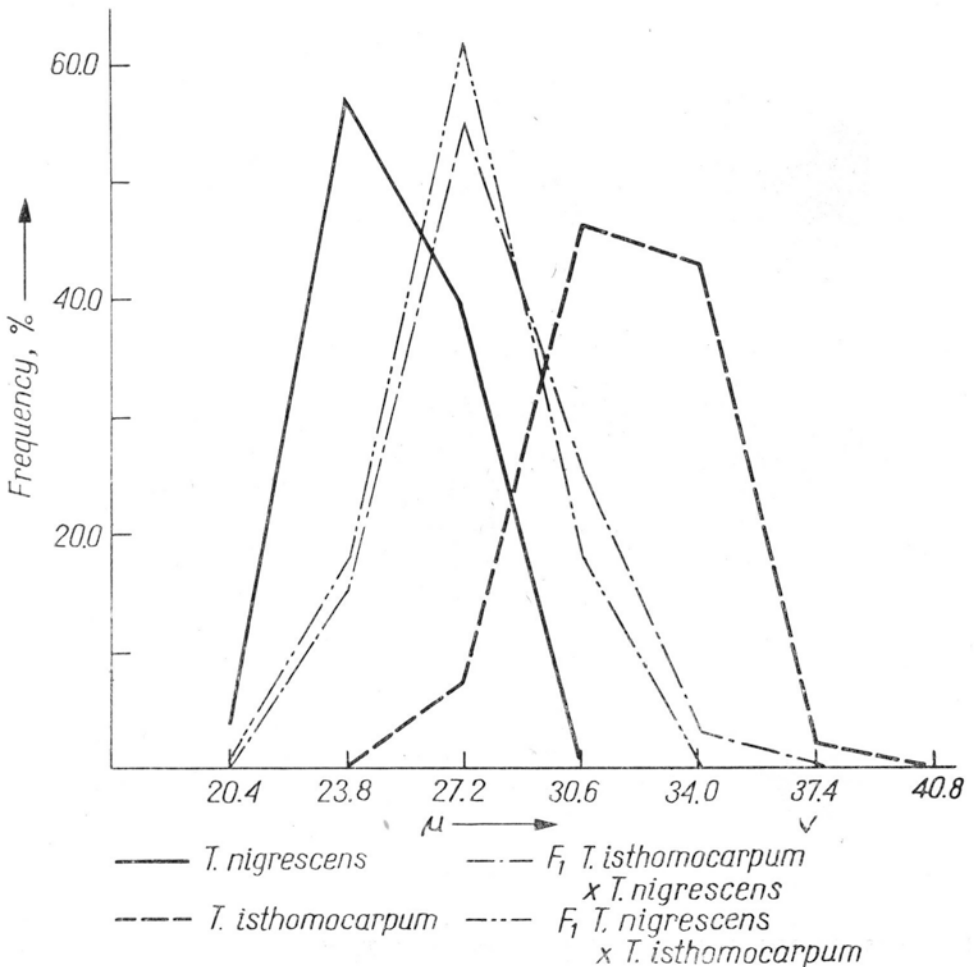
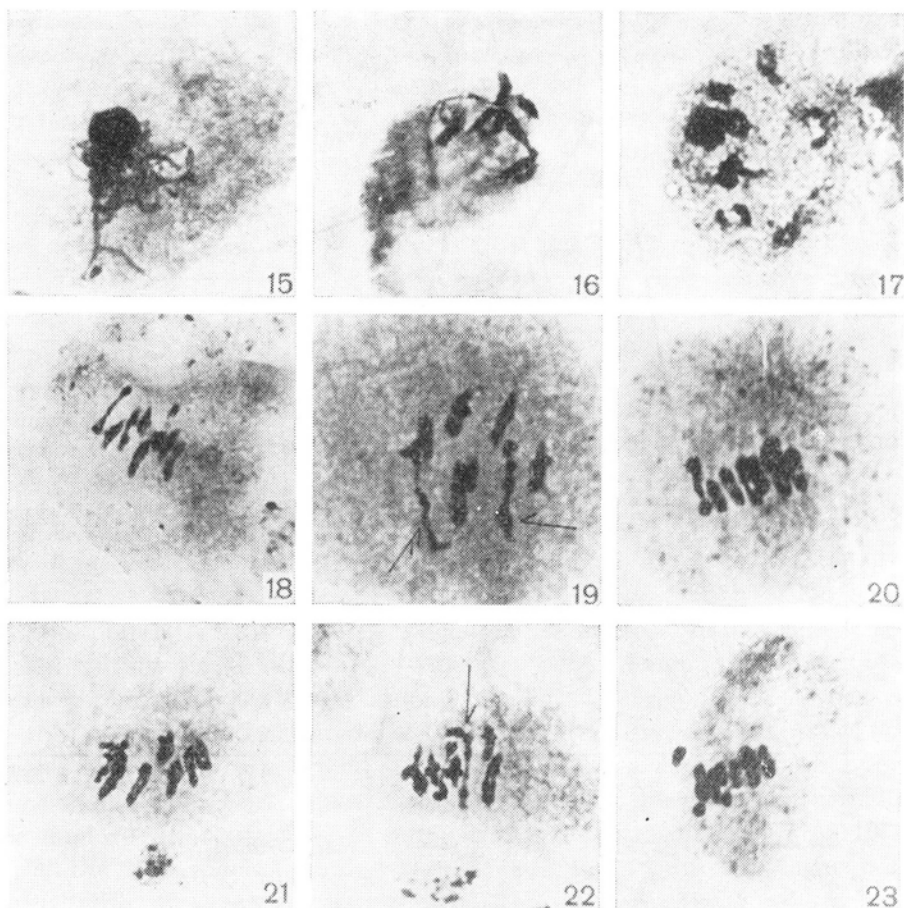


Diagram 1. Variability of pollen grain diameter ( $\mu$ ) in *T. isthomocarpum*, *T. nigrescens* and  $F_1$  (average grain diameter for *T. isthomocarpum* 31.9  $\mu$ , for *T. nigrescens* 21.4  $\mu$ , for  $F_1$  of cross *T. isthomocarpum* × *T. nigrescens* 27.7  $\mu$  and  $F_1$  of the reverse cross 27.2  $\mu$ ).

## Meiosis in the hybrids

The pollen mother cells in the prophase of meiosis are mononuclear and mononucleolar in the hybrids. Only sporadically during diplotene binucleolar cells were found. In the pachytene phase shorter and longer segments of chromosomes joined in bivalents did not conjugate, besides, a single tetravalent was observed indicating translocation in one of the crossed species (Photo 15).

During early diakinesis the chromosomes exhibited high spiralization, in some cells, however, beside the normal spiral one, a single long, hardly shortened bivalent resembling in shape the bivalent in pachytene was observed (Photo 16). From among the 174 pollen mother cells



Photos 15—23. Meiosis in  $F_1$  *T. isthomocarpum*  $\times$  *T. nigrescens* and  $F_1$  *T. nigrescens*  $\times$  *T. isthomocarpum*

15 — Pachytene, tetravalent and a short not-conjugating segments are visible; 16 — Late diakinesis,  $8_{II}$ , poor condensation of one of the bivalents; 17 — Diakinesis,  $6_{II}^1 1_{IV}$ ; 18 — Metaphase I,  $8_{II}$ ; 19 — Metaphase I,  $8_{II}$ , heteromorphic bivalent (arrow), poor condensation bivalent (double arrow); 20 — Metaphase I,  $6_{II}^1 1_{IV}$ ; 21 — Metaphase I,  $6_{II}^1 1_{IV}$ ; 22 — Metaphase I,  $6_{II}^1 1_{IV}$ , heteromorphic bivalent (arrow); 23 — Metaphase I,  $7_{II}^2 1$

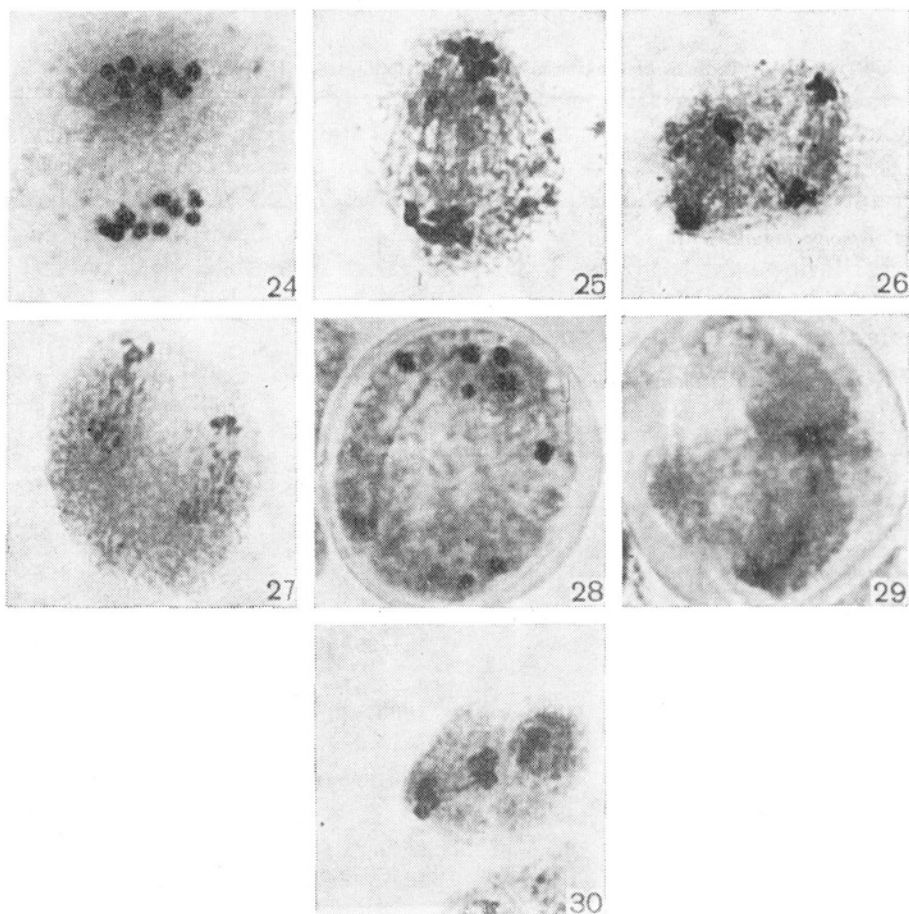
Table 11

Course of diakinesis and first metaphase in F<sub>1</sub> plants

Species	Chromosome figures				No. of cells examined
	II	III	IV	I	
	Diakinesis				
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	8	—	—	—	61
	7	—	—	2	9
	6	—	1	—	86
	5	—	1	2	7
	4	—	2	—	1
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	8	—	—	—	2
	6	—	1	—	6
	5	—	1	2	2
Total	First metaphase				174
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	8	—	—	—	161
	7	—	—	2	19
	6	×	1	—	98
	6	—	—	4	1
	6	1	—	1	1
	5	—	1	2	8
	4	—	2	—	2
	5	1	—	3	1
	5	—	—	—	—
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	8	—	—	—	181
	7	—	—	2	22
	6	—	1	—	119
	6	—	—	4	1
	5	—	1	2	12
	5	2	—	—	1
	4	—	2	—	3
	4	—	—	—	—
Total					630

examined 63 (36%) contained only bivalents, 92 (52%) six bivalents and one tetravalent (Photo 17), and there also were some few cells in which bivalents and univalents were present, of the latter not more than two per cell (Table 11).

The chromosome figures during the first metaphase were similar to those observed in the phase of diakinesis (Table 11). Most numerous among the cells analysed (54%) were those with 8 bivalents (Photos 18 and 19). Cells with 6 bivalents and 1 tetravalent (Photos 20—22) were also numerous (more than 34%). Cells containing bivalents beside univalents were not numerous (Photo 23), the number of the latter did not exceed four in them. Trivalents were detected only in three of the cells examined, one in each of two cells and two in the third cell.



Photos 24—30. Meiosis and sporogenesis in  $F_1$

24 — Anaphase I, 8 chromosomes at two poles; 25 — Late anaphase I, lagging chromosome and late dividing bivalent; 26 — Telophase II, lagging fragment and chromosome, and a late divided bridge originated during the first division; 27 — Anaphase-telophase II, lagging chromosomes; 28 — Prophase in the pollen grain, 8 chromosomes; 29 — Telophase in pollen grain — two lagging chromosomes left after breaking of bridges; 30 — Telophase, chromatide bridge in the perianth somatic cell.

In some cells the bivalents differed in length during the first metaphase. These differences were due to the unequal degree of condensation (Photo 19). In some of the cells examined in this phase of division one heteromorphic bivalent was found (Photos 19 and 22).

Chromosome separation to the opposite poles during first anaphase had in 31 per cent of the cells a normal course, eight chromosomes moved to each pole (Photos 24, Table 12). In the remaining cells a bivalent dividing late (Photo 25) a bicentric bridge and 1 to 2 lagging chromosomes were found. Only in three of the cells studied chromosome separation to the opposite poles was unequal — with nine translocating to one pole and 7 to the other (Table 12). During telophase and inter-

Table 12

Course of first anaphase in F<sub>1</sub> plants

Species	Number of chromosomes			No. of cells examined
	1-st pole	retarded chromosomes and other deviations	2-nd pole	
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	8	—	8	78
	7	late dividing bivalent	7	1
	8	1	7	2
	7	2	7	1
	8	2	6	1
	8	bicentric bridge	8	1
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	8	—	8	45
	8	1	7	1
	9	—	7	3
	8	bicentric bridge	8	2
Total				135

Table 13

Number of chromosomes during second metaphase in F<sub>1</sub> plants

Species	No. of chromosomes in		Remarks	No. of cells examined
	1-st group	2-nd group		
F <sub>1</sub> <i>T. isthomocarpum</i> × <i>T. nigrescens</i>	8	8		190
	8	8	bridge between plates	2
	8	7	1 chromosome outside group	1
F <sub>1</sub> <i>T. nigrescens</i> × <i>T. isthomocarpum</i>	8	8		25
	Total			

phase after the first division only one of the 358 cells analysed (0.28%) contained beside nuclei one additional micronucleus.

The second metaphase had a normal course in the hybrids, there were 8 chromosomes in each of the two groups (Table 13). The only deviations were a bridge between the plates — a remnant of the bivalent not divided during the first anaphase, and in one cell a chromosome lying outside the division plate.

During the second anaphase, cells with retarded chromosomes were found only sporadically and a single cell with a chromatide bridge. With

Table 14  
Course of second anaphase in  $F_1$  plants

Species	Cell number		Kind of deviation
	normally dividing (8 chromosomes in each group)	deviations	
$F_1$ <i>T. isthomocarpum</i> $\times$ <i>T. nigrescens</i>	30	1 1 1	3 retarded chromosomes 1 fragment and 1 retarded chromosome 2 retarded chromosomes
$F_1$ <i>T. nigrescens</i> $\times$ <i>T. isthomocarpum</i>	36	2 1 1	1 retarded chromosome 2 retarded chromosomes bridge

Table 15  
Microsporogenesis in  $F_1$  plants

No. of plant	Kind of polyad						Total
	tetrads	pentads	hexads	septads	octads	nonads	
183/71	207	1					208
9/71	204	1					205
266/71	210	3					213
235/71	230	1					231
233/71	205	2					207
230/71	197	5	2				204
234/71	215	3	2				220
231/71	90	69	43	17	13	2	234

these exceptions the chromosomes migrated regularly in groups of eight to the opposite poles (Table 14).

In the cells examined in the stage of second telophase and interphase mostly four nuclei were present. Retarded chromosomes and micro-nuclei in the number of one to three were noted in some few cells (Photo 26). Only in one plant from the cross *T. nigrescens*  $\times$  *T. isthomocarpum* (plant 231), in which in the process of microsporogenesis more than 60 per cent of various microspore polyads differing from tetrads formed during the second anaphase and telophase, separated and unseparated chromosomes in both the separating groups were scattered along the entire spindle (Photo 27).



After the second division, in seven of the eight  $F_1$  plants of both-way hybrids examined, only few pollen mother cells transformed into microspore polyads differing from tetrads, while the majority of cells formed regular tetrads of microspores (Table 15).

Analysis of mononuclear microspores demonstrated that, from among the 349 cells examined, thirteen (3.7%) contained beside the nucleus a micronucleus, and in each of three cells (0.8%) there were two micronuclei. The remaining 333 cells contained only one nucleus.

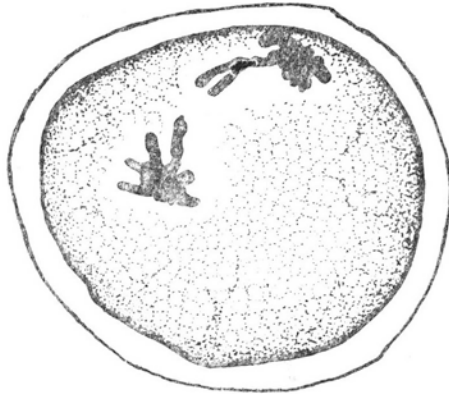


Fig. 1. Telophase in pollen grain from  $F_1$  plant — two retarded chromosomes left after breaking of bridges

During late prophase and metaphase chromosomes were counted in 49 cells of mononuclear pollen. In each of them 8 chromosomes were found (Photo 28). During telophase, however, pollen cells were observed with a retarded chromosome and also cells with one or two broken bridges (Photo 29, Fig. 1). In the meristematic cells of the calyx and perianth, during mitotic anaphase and telophase chromatide bridges were also found (Photo 30).

#### DISCUSSION

Both-way crosses between *Trifolium nigrescens* and *T. isthomocarpum* prove that these species can be crossed in both ways. The large number of seeds obtained and the relatively high per cent of ovules which after fertilization produced hybrid seeds indicate that the species crossed, in spite of wide morphological differences, exhibit a cross compatibility. The compatibility between these species does not decide of the possibility of obtaining viable hybrid plants, this depending on the direction of the cross.

$F_1$  seeds of the *T. isthomocarpum*  $\times$  *T. nigrescens* cross germinated well and the seedlings were green. In the reverse cross, although seed

setting was better than in the other combination (Table 1), the hybrid seeds germinated worse. From among the  $F_1$  seedlings of *T. nigrescens*  $\times$  *T. isthomocarpum* 79.1 per cent were albinotic and died two to three weeks after seeding. The remaining 20.9 per cent proved in most cases to be chlorophyll chimaerae (segmental and periclinal), and only few produced chlorophyll normally. Thus, although the two species investigated did not form a barrier making intercrossing impossible, a different obstacle formed in the genome of *T. nigrescens*, which, though incomplete, made survival of the seedlings and  $F_1$  plants impossible when *T. nigrescens* was used as maternal plant. This obstacle is the inability of chlorophyll production.

The authors have encountered a similar phenomenon earlier when investigating hybrids between *T. nigrescens* and *T. repens* (Kazimierski and Kazimierska, 1970). For explaining the different behaviour of plastids in the reciprocal crosses, the hypothesis has been advanced of differences in the physiological state of plastids in the ovule before fertilization. Namely, according to Menke (1960), there are structures inside the plastids, which require for their synthesis, beside enzymes and substrate, a certain small amount of a reacting product playing the role of initiator. In other cases the reaction occurs without this product.

The incompleteness of the above mentioned obstacle in *T. nigrescens* which makes survival of the  $F_1$  seedlings of the cross *T. nigrescens*  $\times$  *T. isthomocarpum* impossible consists in the appearance among the albinotic seedlings of chlorophyll chimaerae (segmental and periclinal) and of sporadic normal green plants. This phenomenon may arise in a population consisting of heterogenic individuals as regards the genes conditioning chlorophyll synthesis. All the *T. nigrescens* plants exhibited such a heterogenicity, since in the progeny of each cross with *T. nigrescens* as maternal form and *T. isthomocarpum* as paternal most of the seedlings were deprived of chlorophyll, and the chlorophyll chimaerae and green plants appeared with a frequency of 0 to 2 per cross. Seedlings and mottled plants with mosaic leaves which as a rule died before flowering were found in the progeny of two crosses.

The appearance in the crossing combination with *T. nigrescens* as maternal and *T. isthomocarpum* as paternal plant of a small per cent of chlorophyll chimaerae and plants with green leaves beside the albinotic seedlings might also be explained by the development of such individuals along the androgenic pathway of parthenogenesis. Individuals, however, which would be formed as the result of androgenic parthenogenesis should have in their somatic cells a haploid chromosome number ( $2n=n=8$ ). The  $F_1$  plants of *T. nigrescens*  $\times$  *T. isthomocarpum* — chlorophyll chimaerae and those with green leaves — had in the meristematic cells of the growth zone of the root a  $2n=16$

chromosomes, thus they did not arise by way of androgenic parthenogenesis. No other genesis of  $F_1$  plants, in the cross *T. nigrescens*  $\times$  *T. isthmo-carpum*, which are not chlorophyll aberrants — of androgenic diploid parthenogenesis — is possible, since the course of meiosis of two-way hybrids was equal. Thus, only the hypothesis of differences in the physiological state of plastids in the ovule before fertilization in the species crossed and the heterogenicity of the ovules in reference to the genes conditioning chlorophyll synthesis within the individual could explain the appearance in each  $F_1$  cross of *T. nigrescens*  $\times$  *T. isthmo-carpum* of albinotic seedlings, chlorophyll chimaerae and green plants.

During pachytene, on the conjugating chromosome arms short segments were observed within which the chromosomes did not conjugate. During the first metaphase heteromorphic bivalents were found. The appearance of these bivalents and nonconjugating segments is evidence, according to John and Lewis (1965), of deficiency of a part of the chromosome arms in one of the species crossed.

In the majority of the pollen mother cells examined, the bivalents in the phase of diakinesis and first metaphase were highly condensed, in some cells, however, single bivalents showed poor condensation.

The species investigated also differ as regards translocation, since during pachytene, diakinesis and the first metaphase, in a part of the pollen mother cells (42,7%) one and sometimes even two quadrivalents were found. Besides the latter, two univalents were detected (in 10%) and four univalents (in 0.2%) in each of the two pollen mother cells. Sporadically, during first metaphase single trivalents were found. From among the pollen mother cells examined in the phase of diakinesis and first metaphase in as many as 405 (50.2%) cells all the chromosomes were joined in bivalents. On the average for diakinesis and first metaphase the number of bivalents per cell was  $7.014_{II}$ , of trivalents  $0.005_{III}$ , of quadrivalents  $0.435_{IV}$  and of univalents  $0.209_I$ . The first anaphase, with the exception of some few cells, in which a bridge was found, and lagging single chromosome, has in most cells a normal course. After the first division, with the exception of sporadic cases, regularly eight chromosomes were regularly found at each pole. The second division occurred without major disturbances and the pollen mother cells transformed to regular tetrads of microspores, pentads and hexads in seven of the plants examined were found not more frequently than 1 per 74 quartets analysed. Only in one of the  $F_1$  plants (plant 237/71) 60.8 per cent of the pollen mother cells examined after the second division transformed to different from tetrads microspore polyads.

The pollen grains, as shown by calculation of the number of chromosomes in the metaphase plates during division of the pollen grain cell nucleus into a vegetative and a generative one, contain regularly 8 chromosomes each. Although in some grains, in which the dividing

nucleus was in the stage of telophase, one and even two chromatid bridges were found. These bridges during telophase were sometime also detected in the dividing meristematic cells of the perianth.

In the further process of growth and pollen maturation most of the grains died, and in the anthers of developed  $F_1$  flowers there were only 3.1—17.0 per cent of fertile pollen grains.

The hybrids under investigation did not set seeds after self-pollination and when crossed between themselves and pollinated with pollen of the parental forms they gave only few seeds. On the other hand, the parental forms pollinated with  $F_1$  pollen set seeds quite well. The poor setting of seeds in the intercusses between  $F_1$  plants and in back crosses shows that the per cent of fertile female gametes in the hybrid was many times lower than the per cent of fertile pollen grains. Thus, the observation of Poddubnaya - Arnoldi (1964) that the female gametophyte becomes sterile more rarely and to a smaller extent than the male one, probably because it is better protected from the direct action of external conditions, since it is covered with the tissues of the ovule and ovary, does not find confirmation in the case of the hybrid in point and other clover hybrids (Kazimierski and Kazimierska, 1968, 1970; Kazimierski, Kazimierska and Strzyżewska, 1972) of some *Secale* hybrids (Khush, 1962, 1963; Khush and Stebbins, 1961), of *Lolium* (Rudelle and Essad 1968) and other plants.

#### CONCLUSIONS

1. *T. nigrescens* crosses with *T. isthomocarpum* reciprocally. The hybrid seeds are well filled; their germination and viability of the seedlings depend on the direction of the cross. Seeds of the cross *T. isthomocarpum* ♀ × *T. nigrescens* ♂ germinate better and give green seedlings, while seeds of the reverse cross germinate poorly and the seedlings are mostly albinotic.

2. Normal chlorophyll production by  $F_1$  plants of the *T. isthomocarpum* × *T. nigrescens* cross and the albinism of most  $F_1$  plants from the reverse cross may be explained by different physiological states of the ovules before fertilization of these two species (Menke, 1960). Sporadic appearance among the  $F_1$  crosses of *T. nigrescens* × *T. isthomocarpum* of seedlings and plants which are chlorophyll chimaerae (segmental, periclinal) and plants with mottled leaves as well as green normal plants is conditioned by the heterogenicity of the genes conditioning chlorophyll synthesis in *T. nigrescens* plants.

3. The species investigated differ in translocation and small deficiencies. In 42.7 per cent of pollen mother cells examined in the stage of first metaphase one and even two quadrivalents were present. On the average for pollen mother cells in the stage of diakinesis and first

metaphase the number of bivalents per cell was 7.014<sub>II</sub>, of trivalents 0.005<sub>III</sub>, of quadrivalents 0.435<sub>IV</sub> and of univalents 0.209<sub>I</sub>. For one half of the pollen mother cells in the first metaphase the typical chromosome arrangement was 8<sub>II</sub>. The first and second divisions occurred almost normally. After the second division, in most of the F<sub>1</sub> plants, almost exclusively microspore tetrads are formed. The F<sub>1</sub> plants produced 3.1—17.0 per cent of fertile pollen grains.

4. After selfpollination the F<sub>1</sub> gave no seeds. Crossed between themselves and backcrossed they set but few weak seeds. On the other hand, the parental plants pollinated with F<sub>1</sub> pollen set seeds normally. Thus, the fertility of the ovules in F<sub>1</sub> plants was many times lower than that of the pollen.

5. Morphologically the F<sub>1</sub> plants were closer to *T. nigrescens* than to *T. isthomocarpum*. The hybrids exhibited the following dominating traits derived from *T. isthomocarpum*: the intensive colouring of the stems, petioles and sepals with anthocyanin and the greater number of florets in the head. The dominating traits inherited from *T. nigrescens* and visible in F<sub>1</sub> plants was the setting of flowers in the head on long peduncles and their drooping after the end of flowering.

6. The ready crossing between *T. nigrescens* and *T. isthomocarpum* indicates a close relationship of these species. The almost complete sterility of the hybrids after selfpollination and cross pollination results from changes in chromosome structure (translocation, small deficiencies) which have accumulated in the chromosomes of the species studied in the course of evolution.

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### Badania mieszańców w rodzaju *Trifolium* L. V.

#### Streszczenie

Zbadano krzyżowanie się *T. isthomocarpum* z *T. nigrescens* oraz przebieg mejozy i płodność u otrzymanych mieszańców. Badane gatunki krzyżują się przeciwnie. Nasiona mieszańcowe są dobrze wypełnione, a kiełkowanie nasion i przeżywalność siewek zależą od kierunku krzyżowania. Nasiona krzyżówki *T. isthomocarpum* ♀ × *T. nigrescens* ♂ kiełkują lepiej i dają siewki zielone, nasiona krzyżówki *T. nigrescens* ♀ × *T. isthomocarpum* ♂ kiełkują gorzej, a siewki są przeważnie albinotyczne.

Normalne wytwarzanie chlorofilu przez rośliny  $F_1$  *T. isthomocarpum* × *T. nigrescens* i albinotyczność większości siewek krzyżówki odwrotnej można tłumaczyć różnym stanem fizjologicznym komórek jajowych przed zapłodnieniem u dwóch krzyżowanych gatunków. Sporadyczne pojawianie się wśród krzyżówek  $F_1$  *T. nigrescens* × *T. isthomocarpum* siewek i roślin chimer chlorofilowych (sektorialnych, periklinalnych i roślin o liściach marmurkowych) oraz roślin normalnie zielonych warunkowane jest heterogenicznością genów warunkujących syntezę chlorofilu u roślin *T. nigrescens*.

Badane gatunki różni translokacja i drobne definicjencje. W 42,7% komórek macierzystych pyłku w stadium pierwszej metafazy znajdowano 1, a nawet 2 kwadriwalenty. Przeciętnie dla komórek w stadium diakinezy i pierwszej metafazy, liczba bivalentów wynosiła 7,01<sub>II</sub>, triwaleńców — 0,005<sub>III</sub>, kwadriwaleńców — 0,435<sub>IV</sub>

oraz uniwalentów — 0,209<sub>1</sub>. Dla połowy analizowanych komórek macierzystych pyłku typowym układem chromosomów było 8<sub>II</sub>. Pierwszy i drugi podział przebiegały bez mała normalnie. Po drugim podziale u większości z badanych roślin F<sub>1</sub> formowały się prawie wyłącznie tetrazy mikrospor. Rośliny F<sub>1</sub> produkowały od 3,1 do 17,0% żywotnych ziarn pyłku.

Rośliny gatunków rodzicielskich były samoniezgodne; mieszańce F<sub>1</sub> po samozapyleniu także nie dały nasion. Krzyżowane między sobą i wstecznie z roślinami gatunków rodzicielskich wiązały niewiele słabych nasion. Natomiast rośliny gatunków rodzicielskich zapylane pyłkiem z roślin F<sub>1</sub> wiązały nasiona dobrze. Zatem żywotność komórek jajowych u roślin F<sub>1</sub> była wielokrotnie niższa niż żywotność pyłku.

Morfologicznie mieszańce były bliższe *T. nigrescens* niż *T. isthomocarpum*. Ze strony *T. isthomocarpum* dominowały u mieszańców następujące cechy: intensywne zabarwienie antocyjanem łodyg, ogonków liściowych i działek kielicha, dłuższy kwiat, wrzosowe zabarwienie korony kwiatowej i większa liczba kwiatów w główce; ze strony *T. nigrescens* u F<sub>1</sub> dominowała cecha osadzenia kwiatów w główce — na długich szypułkach kwiatowych i zwisanie ku dołowi kwiatów po przekwitnięciu.

Łatwe krzyżowanie się *T. nigrescens* z *T. isthomocarpum* i przebieg mejozy u F<sub>1</sub> wskazują na bliskie pokrewieństwo badanych gatunków. Prawie kompletna bezpłodność mieszańców wynika ze zmian w strukturze chromosomów (translokacja, drobne deficjencje), jakie nagromadziły się w chromosomach badanych gatunków w procesie ewolucji.