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Morphology and fertility of a spontaneous octoploid of white clover (*Trifolium repens* L.)

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Abstract. In the progeny of a hybrid obtained after crossing local Polish and local Bulgarian white clover genotypes one octoploid (2n=64) plant was selected. In view of difficulties to obtain seeds, the octoploid plant was vegetatively propagated. The progeny of the octoploid obtained as a result of self- and cross-pollination of cloned plants was examined and compared to related tetraploids with respect to morphological characters, fertility and embryo sac structure. Vegetative and generative organs of the octoploid were slightly larger than those of tetraploids. The number of flowers per head was larger in the octoploid than in tetraploid plants. The spontaneous octoploid appeared to have a low fertility after both cross- and self-pollination. It was most probably caused by low pollen viability, by decline of megasporocyte and megagametocyte in the process of ontogenesis and by a smaller ovule number per ovary. Seeds of octoploids were partially underdeveloped and only 23.85% of them gave rise to seedlings.

Key words: fertility, morphology, spantaneus octoploidy, white clover.

Trifolium repens is a tetraploid species with the chromosome number 2n=4x=32 (ERITH 1924, DARLINGTON, WYLIE 1955). Octoploids (2n=64) of white clover known so far were obtained by colchicine treatment (MACKIEWICZ 1970a,b,c). It has appeared that they were of little use for cultivation.

In the present paper the spontaneous octoploid of *T. repens* selected from the progeny of a hybrid between local Polish and local Bulgarian genotypes is described in respect of morphological characters, fertility and embryo sac structure.

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Material and methods

Material for the studies covered the octoploid of white clover found in the progeny of the hybrid derived after crossing local Polish and local Bulgarian genotypes, as well as tetraploids related to it. In view of difficulties to obtain seeds, the octoploid plant was vegetatively propagated. Progeny of the octoploid obtained after spontaneous self-pollination, artificial self-pollination and cross-pollination was examined for some morphological characters, fertility and embryo sac structure.

Roots for cytological analyses were fixed in Carnoy's solution. The chromosome number in cells was determined on squashes stained with acetocarmine.

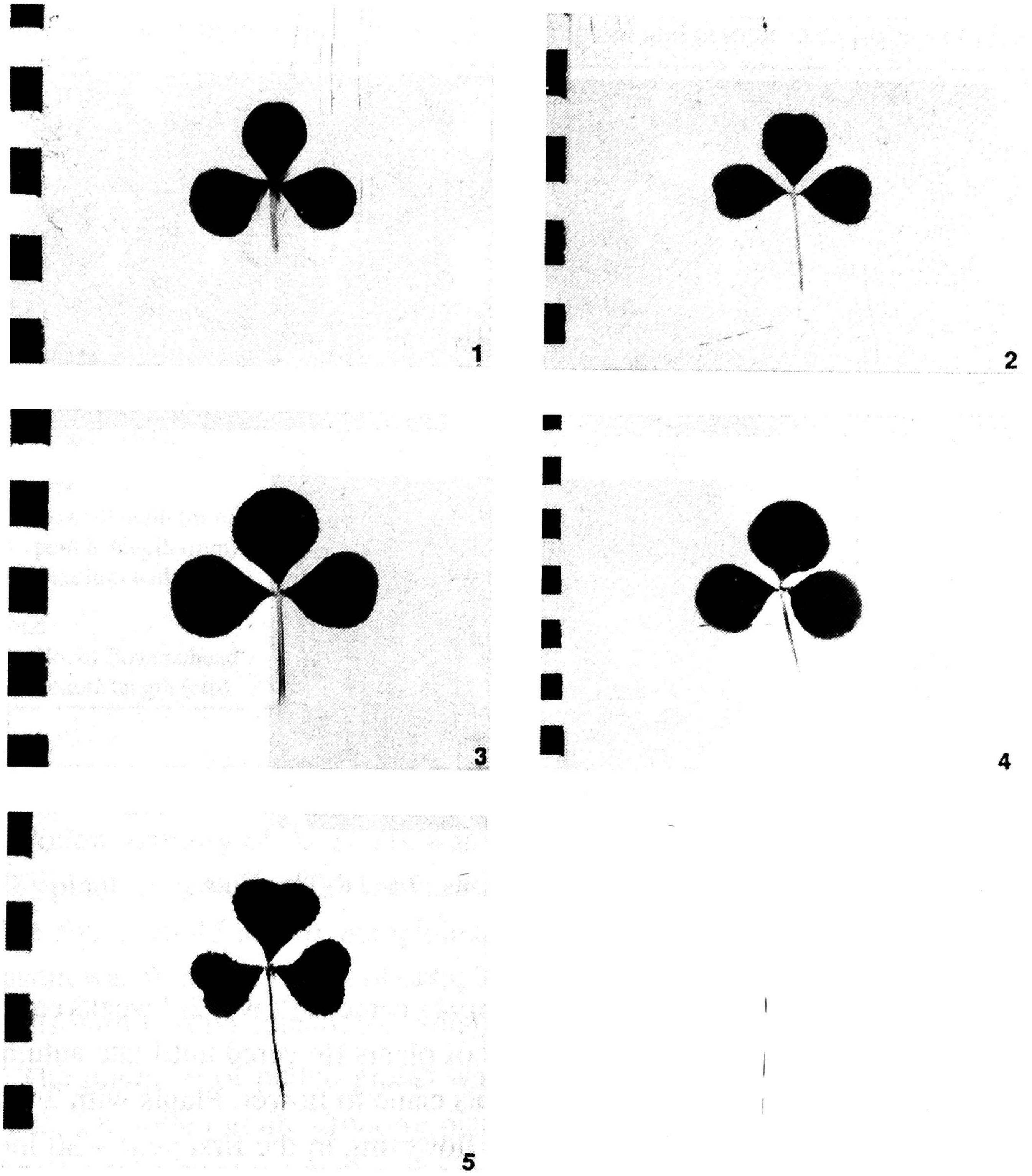
For an analysis of the structure and development of female gametophyte the ovaries were fixed in Navashyn's mixture. After dehydration they were embedded in paraffin, cut on a microtome, stained with iron hematoxylin, cell walls were tinted with fast green and through xylen were enclosed in Canada balsam.

Pollen grain viability and diameter were determined on preparations stained in Belling's solution. In the vegetation period, the beginning of plant flowering was noted, the flower length and vexillum width were measured and the number of flowers in developed inflorescences was counted. The length and width of stoma cells as well as their number per leaf area unit were determined on the cuticle taken from the bottom side of leaf and placed into Belling's solution.

Plant fertility was studied after spontaneous self-pollination, artificial pollination, i.e. after carina break and transfer of plant's own pollen on the stigma, as well as after cross-pollination. In mature fructifications, pollinated in different ways, pods and seeds were counted.

Results

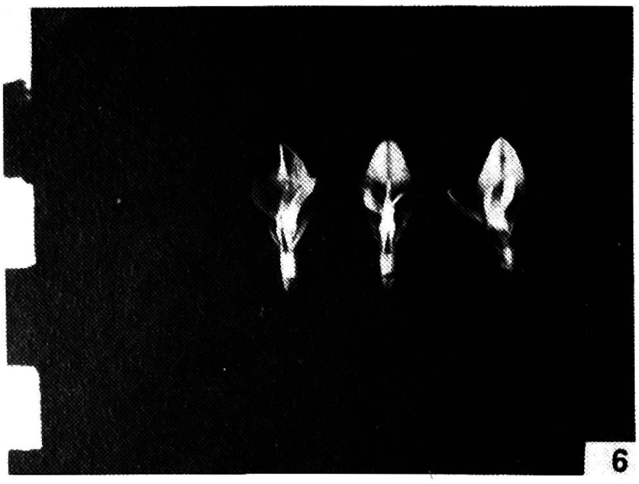
Octoploid white clover developed two inflorescences with few flowers; after self-pollination it set no seeds and for that reason it was cloned for reproduction. Next year, after breaking carina by hand and pollen transfer on the stigma, 39 pods were set in 185 pollinated flowers, from which 34 seeds were obtained. The seeds gave rise to 20 plants with coarse, intensively green leaves and dark-pink flowers. In all these plants 64 chromosomes were found in the root tip cells, which indicated that they were octoploids.



Figs. 1-5. Leaves: 1 – 4x plants; 2-5 – 8x plants

Leaflet blades of these plants were slightly longer than those of normal *T. repens* plants, but did not differ in width (Table 1). However, within octoploid plants, leaflet blades differed in size and shape (Figs. 1-5).

The length and width measurements of stoma cells as well as their number per leaf area unit show that in octoploids these cells were 10 μm longer and 6-7 μm wider, while their number per leaf area unit was almost twofold smaller than that in tetraploid plants (Table 1).



Figs. 6-8. Flowers: 6 – 4x plants; 7 and 8 – 8x plants

Plants with the chromosome number 2n=4x came to flower 2-3 weeks earlier than plants with 2n=8x. The both groups of plants flowered until late autumn. In the first year of vegetation, all 4x plants came to flower. Plants with 2n=8x can be divided into three groups: plants flowering in the first year – 30 individuals (63.8%), plants flowering in the second year of vegetation – 4 plants (8.5%) and plants not flowering at all – 13 individuals (27.6%), 4 of which did not survive winter.

Flowers of 4x plants were white, sometimes slightly pink-tinted. In 8x plants they were intensively dark-pink. Flowers of 4x and 8x plants slightly differed in length, being a little longer in 8x than in 4x plants, whereas vexilla of 8x flowers were conspicuously wider than those of 4x plants (Table 1, Figs. 6-8). The flower petioles were somewhat longer than in 4x plants (Table 1).

Inflorescences of 8x plants had, on average, a little more flowers than those of 4x plants (Table 1, Figs. 9-11); inflorescence of the first ones were set on longer stems than those of the second plants.

Table 1. Some morphological characters of tetraploid and octoploid white clover plants

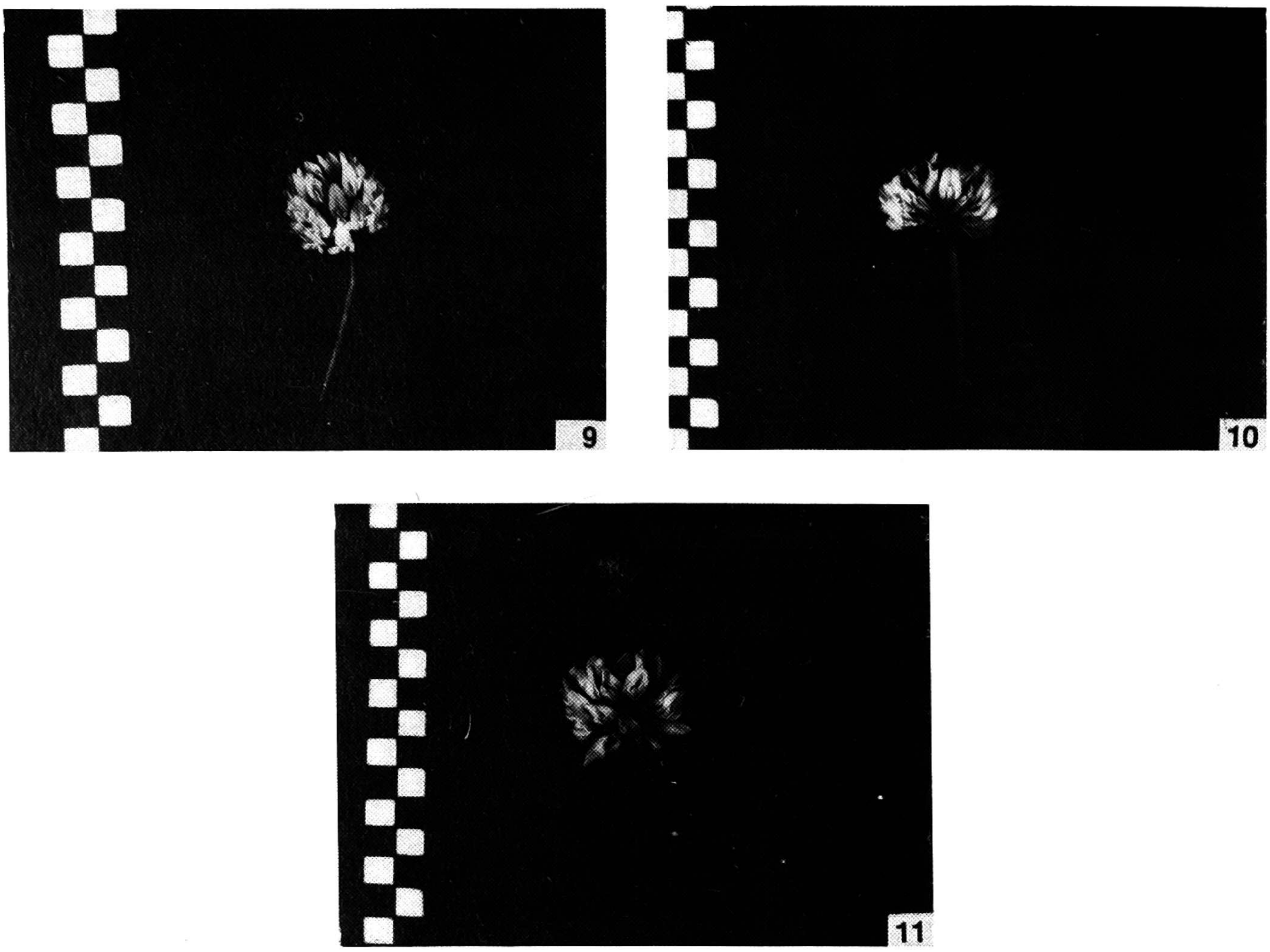
Character	Tetraploid		Octoploid	
	mean	range	mean	range
Leaflets				
length (mm)	12.1	10.0-14.0	14.6	15.0-17.0
width (mm)	11.6	9.0-14.0	12.8	10.0-16.0
length : width	1.03		1.18	
Stoma cells				
length (mm)	22.76	20.4-25.0	32.65	28.2-36.0
width (mm)	15.23	12.6-18.3	22.29	20.3-25.2
length : width	8.5	7.0-11.0	4.1	3.0-6.0
Flowers				
flower length (mm)	9.3	8.0-10.5	10.2	10.0-12.5
petiole length (mm)	1.9	0.5-2.0	2.11	1.0-3.2
vexillum width (mm)	4.2	4.0-5.0	5.7	5.0-6.5
Head				
No. of flowers/head	53.8	27.0-66.0	58.1	17.0-96.0
Petiole length (cm)	15.1	9.0-31.0	16.9	12.0-28.0

Pollen viability of 4x plants was over 90% (Table 2, Fig. 11), while that of 8x plants ranged from 0.0 to over 90%. Pollen grains without plasma (Fig. 13) were found in 15.9% of octoploids. More than 90% of pollen grains with plasma was found in 14.5% of octoploid plants (Fig. 14). In octoploids, pollen grains with plasma constituted from several to 90% (Table 2, Fig. 15).

The diameter of pollen grains was 6.1 μm longer in 8x than in 4x plants (Table 2). Pollen grains from 4x plants were found to have three micropyles of pollen tube outlets, whereas those of 8x plants had mostly four micropyles, though grains with three and five micropyles were sporadically encountered.

Table 2. Viability and diameter of pollen grains in tetraploid and octoploid white clover plants

Character	Tetraploid			Octoploid		
	No. of analysed plants	mean	range	No. of analysed plants	mean	range
Percent of pollen grains with plasma	20	95.6	81.2-99.9	69	57.4	0.0-96.4
Diameter of pollen grains (μm)	20	28.7	25.9-32.2	58	34.8	22.7-47.9



Figs. 9-11. Inflorescences: 9 – 4x plants; 10 and 11 – 8x plants

The fertility of 4x and 8x plants was tested in an isolated greenhouse, separately for both groups after cross-pollination, artificial pollination and spontaneous self-pollination (Table 3). The both groups of plants after spontaneous self-pollination were found to have individuals without seeds, but more plants without seeds were found in the group of 8x plants as compared to 4x plants (Table 3). Among plants, which gave seeds after spontaneous self-pollination the percentage of pods with seeds appeared to be higher in the group of 8x plants.

After hand self-pollination, where after carina break plant's own pollen was transferred on the stigma, pods with seeds were in all individuals from the group of 4x plants, and the seed number per pod ranged from 1 to 5 (Table 3). Half 8x plants after hand self-pollination gave no seeds, and the remaining plants set 1-2 seeds per pod.

It may, therefore, be assumed that 4x plants showed a higher selfcompatibility than 8x plants. All 4x plants after cross-pollination gave 1-3 seeded pods, and the per cent of successful crosses was above 48% (Table 3). Part of 8x plants (55%) gave no seeds after cross-pollination and the remaining plants

Table 3. Seed setting after spontaneous self-pollination artificial self-pollination and cross-pollination in tetraploid and octoploid white clover plants

Plants	Analysed plants (No.)	Pollinated flowers (No.)	Pods with seeds (No.)	Pods with seeds (%)	Seeds per pod (No.)
Spontaneous self-pollination					
tetraploid	4	2059	0	0	0
	16	8444	152	1.79	1-2
octoploid	8	4461	0	0	0
	12	19724	423	2.14	1-2
Artificial self-pollination					
tetraploid	20	603	383	63.51	1-5
octoploid	10	495	0	0	0
	10	707	141	17.47	1-2
Cross-pollination					
tetraploid	10	204	99	48.53	1-3
octoploid	11	393	0	0	0
	9	331	58	17.52	1-2

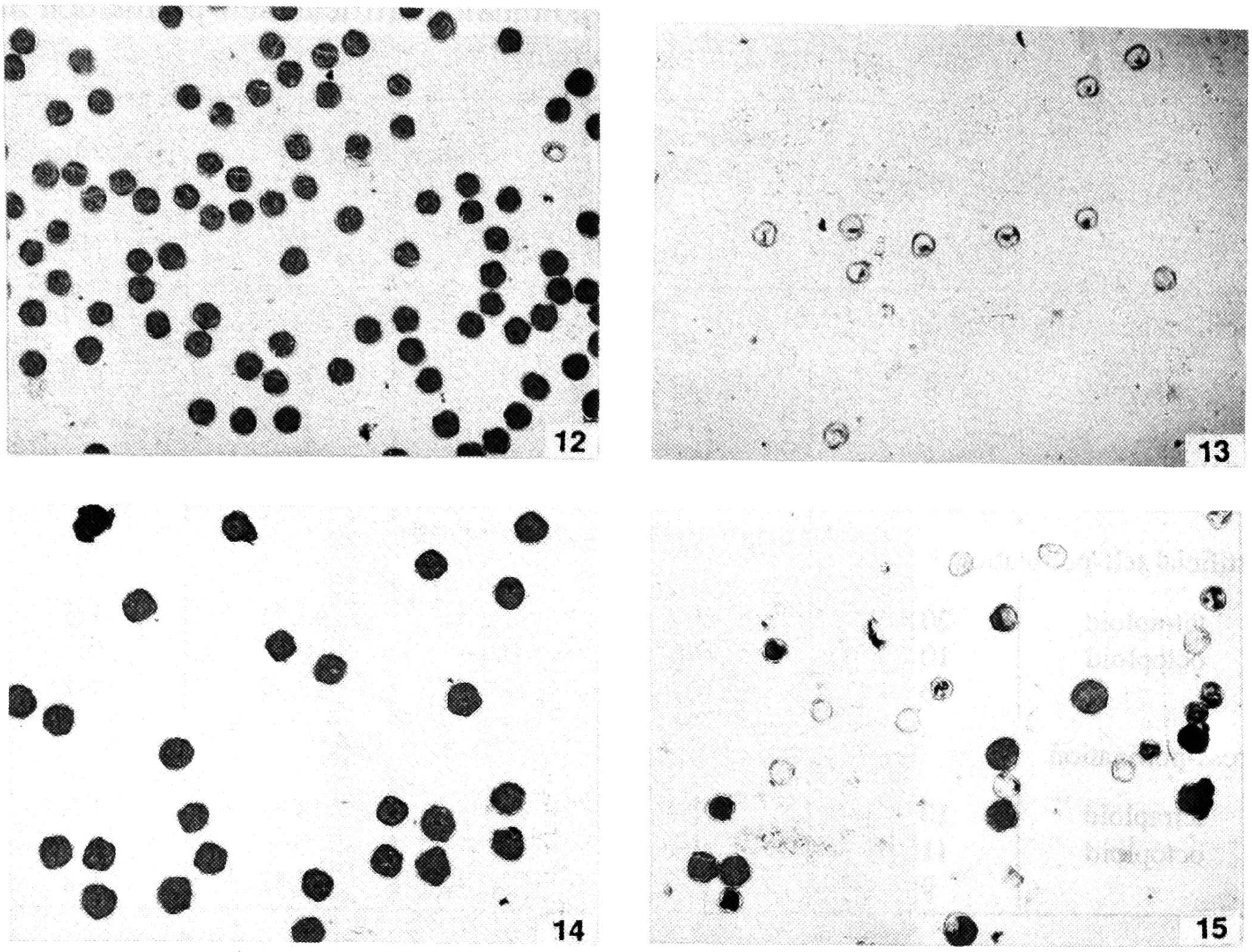
(45%) produced mostly 1-seeded and seldom 2-seeded pods. Intercrossed 8x-plants appeared to be less fertile than 4x plants (Table 3).

Seeds of white clover are hard and in this connection before sowing they were exposed to the processes of scarification. Seeds of 4x plants planted into sterilized soil of a greenhouse gave rise to 98% of seedlings, whereas seeds of 8x plants gave rise to only 23.85% of seedlings. It should be mentioned that part of seeds from 8x plants was less filled. Therefore, their worse germination could be caused by disturbances in the development of embryo.

The ovule of the both studied groups of plants of campylotropous type consists of two integuments – external and internal (Fig. 16 – 4x, Fig. 17 – 8x). The ovule number per ovary ranges from 4 to 6 in 4x plant and from

Table 4. The number of ovaries with 1-6 ovules per ovary in tetraploid and octoploid white clover plants

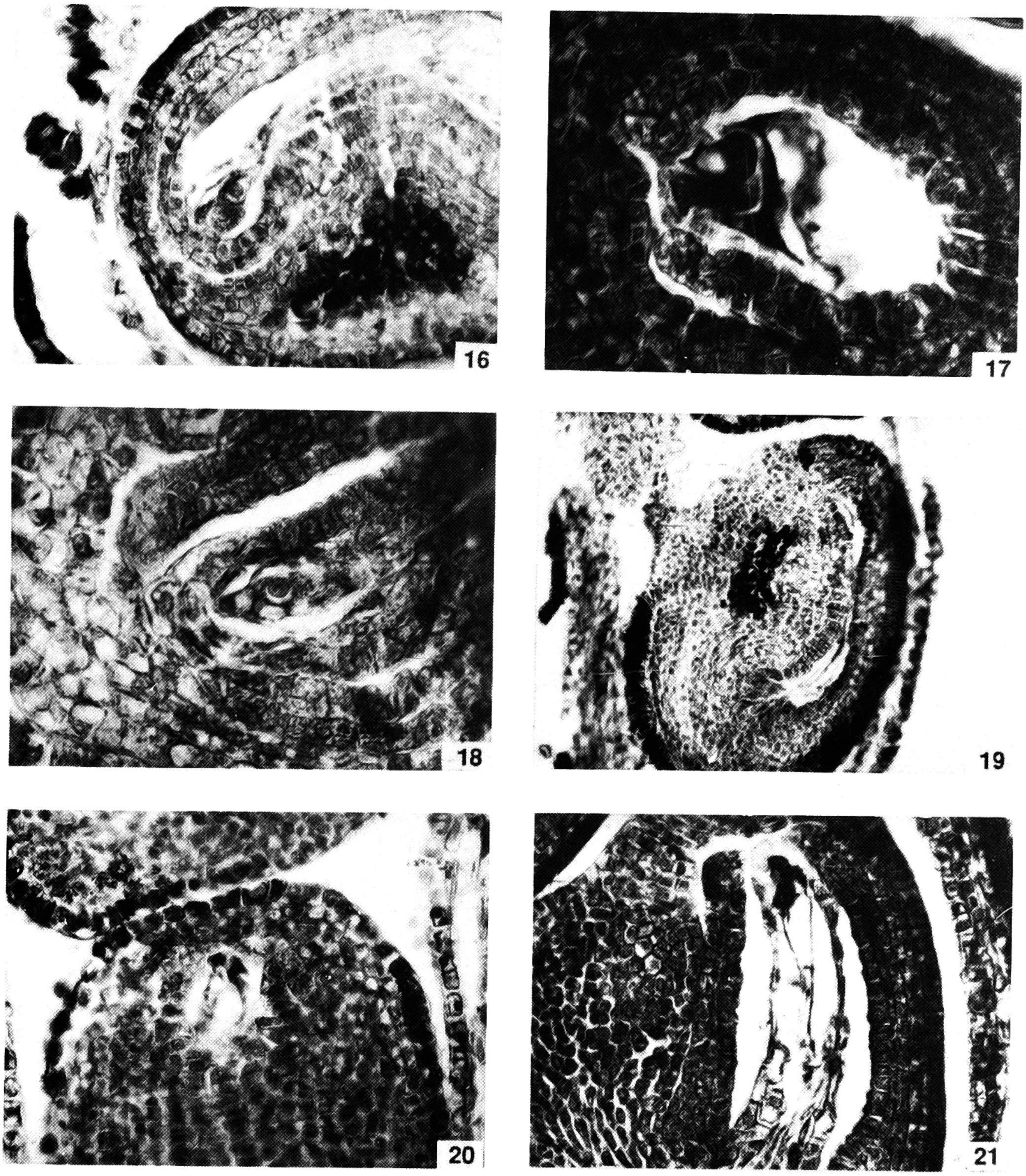
Plants	No. of analysed ovaries	Ovule no. per ovary: 1	2	3	4	5	6	Mean number of ovules per ovary
Tetraploid	15				5	8	2	4.8
Octoploid	70	2	3	19	32	11	3	3.7



Figs. 12-15. Pollen: 12 – 4x plants; 13-15 – 8x plants

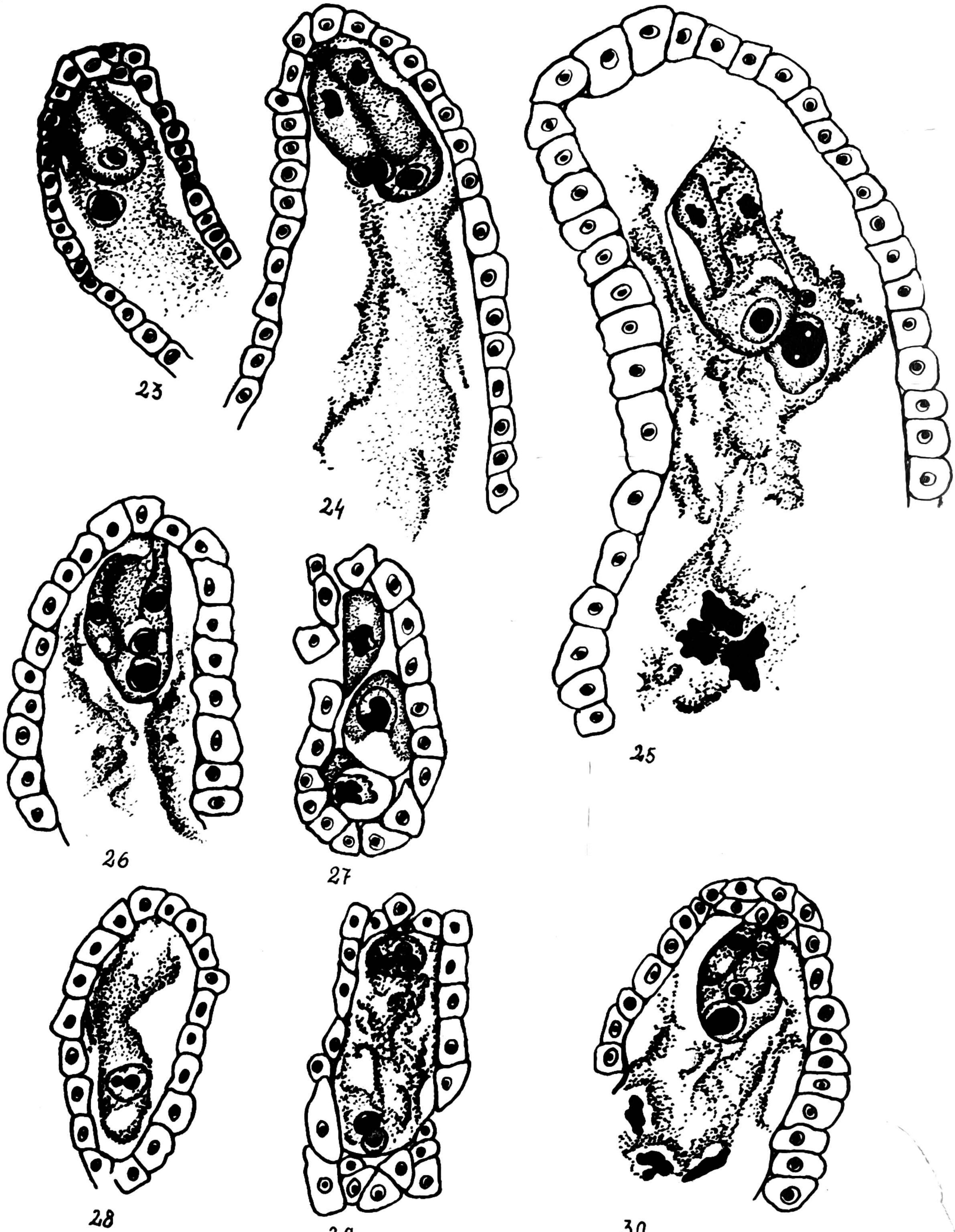
1 to 6 in 8x plants (Table 2). On average, the ovary of 4x form was found to have 1.1 ovules more than that of 8x plant. No deviations was found in the development of the embryo sac of 4x plant. Mature embryo sac had an egg apparatus and the second nucleus in its micropylar end (Figs. 16 and 23). Few embryo sacs of 8x plants were found to have typical egg apparatus (Figs. 17, 26 and 30). Uninucleate embryo sacs were strongly vacuolated (Fig. 18). In other sacs, tetrads and triads of megaspores degenerated (Fig. 27). Division of nuclei was nonsynchronous (Fig. 29), the nucleus of uninucleate embryo sac contained two nucleoli (Fig. 28), and synergid nuclei degenerated comparatively frequently (Figs. 24 and 25). Embryo sacs with a small additional nucleus in the micropylar end and with lately disappearing antipodes in the chalazal end were also encountered (Figs. 19 and 20). In most of ovules the embryo sac was subject to degeneration and was pressed by outgrowing cells of the external integument (Figs. 19 and 20). In few of them, with developed egg apparatus and secondary nucleus, the plasma was coarse-granular, broken by irregular large vacuoli (Fig. 21).

In 8x plants like in 4x plants the anthers consisted of two pollen sacs (Fig. 22).



Figs. 16-21. A longitudinal cross section of ovules of 4x and 8x plants:
16 – 4x plants; 17-21 – 8x plants: 17 – an embryo sac with the egg cell, synergids and priendospermic nucleus; 18 – strongly vacuolated uninucleate embryo sac; 19 – a pressed embryo sac; 20 – degenerating synergids; 21 – a strongly vacuolated sac with degenerating egg apparatus and priendospermic nucleus

Fig. 22. A transverse cross section of anther of 8x plant with two pollen sacs



Figs. 23-30. The embryo sac of 4x and 8x plants of white clover

23 – Mature embryo sac of 4x plants; 24-30 – Embryo sacs of 8x plants; 24 – Degenerating synergids; 25 – Degenerating synergids, antipodes and an additional small nucleus in the vicinity of the egg cell; 26-30 – Matured embryo sac; 27 – A degenerating triad of megaspores; 28 – A uninucleate embryo sac, the nucleus with two nucleoli; 29 – division of nuclei nonsynchronous; 30 – a nonsynchronous division of nuclei.

Discussion

The chromosome number 2n=32 for *T. repens* has been determined for the first time by ERITH (1924). Based on the studies of other clover species and on the data from literature, DARLINGTON and WYLIE (1955) accepted that this species is tetraploid, as the chromosome number for other species of the section Amoria, which *T. repens* was referred to, was 2n=16. Besides that, *T. repens* is crossed, though with difficulty, to other species, and chromosomes of its hybrid form 8 bivalents as a maximum.

Plants of *T. repens* with a double chromosome number (2n=64), i.e. octoploids were obtained many times using colchicine (LEVAN 1942, MACKIEWICZ 1970a). Moreover, white clover is known to have octoploid cells in root warts

Therefore, the found plant with the chromosome number 2n=64 in the somatic cells is an octoploid. Its progeny appeared to be very differential in respect of both morphological traits and its fertility. Flowers of octoploid plants did not differ much in length from those of 4x plants, however, their vexilla were wider than those of tetraploids. The number of flowers per head was larger than that of tetraploids. As reported by MACKIEWICZ (1970c), the average diameter of pollen grains was 2.5 μm larger in octoploids obtained by colchicine treatment than in tetraploids. In the spontaneous octoploid it appeared to by larger by 6.2 μm.

Pollen grain viability of octoploid plants ranged from 0.0 to 96.8%, and 15.7% of individuals without viable pollen were observed. Octoploids studied by MACKIEWICZ (1970a) had no plants without pollen and the average pollen viability amounted to 87.2%. ŁĘCZYŃSKA-HULEWICZ (1965) also found no plants without pollen in tetraploid red clover. The spontaneous octoploid of white clover, therefore, differs significantly from the forms obtained by colchicine treatment in respect of this property.

Tetraploids of red clover are generally less fertile than diploids (ŁĄCZYŃSKA-HULEWICZOWA 1960, JARANOWSKI, KAŁASA 1971, NAVALIKHINA 1973, DE WET 1979). Octoploid of *T. repens* is also less fertile than diploid. However, in the octoploid examined by MACKIEWICZ (1970b, c), the embryo sac developed similarly to that of tetraploid, and pollen viability was only insignificantly lower. The number of ovules per ovary in the both forms was similar. Pollen viability of the octoploid form of *T. repens* under study ranged from 0.0 to 90%. The ovule number per ovary was smaller in 8x than in 4x plant. The embryo sac in octoploid died in most analysed ovules at different stages of ontogenesis. It, may, therefore, be suggested that low fertility of spontaneous 8x plant is caused by irregularities in the process of megaspore and megagamete formation. Irregularities of this kind are frequently encountered in low fertile interspecific hybrids and sometimes in tetraploids obtained by colchicine treatment (PODDUBNAJA-ARNOLDI 1976, RODKIEWICZ 1973). A lower fertility of the studied spontaneous octoploid sustained after all kinds of self-pollination as well as after cross-pollination. It may be suggested that the main reason of a low fertility of this form are irregularities in the development of its embryo sac. Significant part of seeds from octoploid plants germinated poorly. They most probably were underdeveloped as a consequence of disturbances in the formation of egg cell and secondary nucleus.

DAVIS (1966), MACKIEWICZ (1970a) and BIJOK et. al. (1970a,b) report that the pollen head of clover has four pollen sacs. According to KAZIMIERSKI and KAZIMIERSKA (1974), the anther in tetraploid *T. repens* is twosporangiate and octoploid white clover also contains two pollen sacs. Spontaneous duplication of the chromosome number caused no changes in the structure of pollen head.

Conclusions

The spontaneous octoploid (2n=8x=64) of white clover arose from fusion of unreduced gametes or parthenogenetically after duplication of the chromosome number in mother cell of the embryo sac.

An embryological analysis of the development of embryo sacs in the spontaneous octoploid plants showed their decline during ontogenesis. It entails a strong decline in fertility in comparison to tetraploids.

Pollen heads of 8x plants consist of two pollen sacs, spontaneous duplication of the chromosome number has caused no changes in their anatomical structure.

Spontaneous duplication of the chromosome number in white clover entailes a decline in fertility, viability and even a lower stability of plants. It also impoverishes leafage. The spontaneous octoploid is cognitively of interest, though it is difficult to expect that it can be used in practice.

REFERENCES

BIJOK K., GÓRAL M., GÓRAL S. (1970a). Studia embriologiczne nad poliploidalnymi formami *Trifolium repens* L. Acta Agrobot. 23: 259-273.

BIJOK K., GÓRAL M., GÓRAL S. (1970b). Badania embriologiczne di- i tetraploidalnych form *Trifolium hybridum* L. Acta Agrobot. 23: 278-295.

DARLINGTON C.D., WYLIE A.P. (1955). Chromosome Atlas of Flowering Plants. London, George Allen and Uwin. Ltd.: 520.

DAVIS G.L. (1966). Systematic Embriology of the Angiospermes. John Wile and Sons, INC. New York London, Sydney.

De Wet J., (1979). Origins of polyploids. In: Polyploidy Biological Relevance. (H. Levis ed.). Plenum Press. New York and London: 3-15.

Erith A.G. (1924). Withe clover (*Trifolium repens* L.). London Duckworth: 150.

Jaranowski J., Kałasa M. (1971). Comparative analysis of fertility in several *Trifolium, Melilotus, Medicago* and *Trigonella* species and forms on a di- and tetraploid level. Genet. Pol. 12: 1-16.

Kazimierski T., Kazimierska E.M. (1974). Structure of anther heads in some *Trifolium* L. species. Acta Soc. Bot. Pol. XLIII: 321-329.

Levan A. (1942). Plant breeding induction of polyploidy and some reseults in clover. Hereditas 28: 245-246.

Łączyńska-Hulewicz T. (1960). Self-fertility in di- and tetraploid red clover. Zesz. Probl. Post. Nauk Rolniczych 20: 109-111.

Mackiewicz T. (1970a). Microsporogenesis and heterochromatin in octoploid *Trifolium repens* L. Genet. Pol. 11: 241-247.

Mackiewicz T. (1970b). Female gametophyte development in octoploid white clover (*Trifolium repens* L.) Genet. Pol. 11: 37-44.

Mackiewicz T. (1970c). Pollen grain morphology, viability and pollen tube development in vitro and in situ in octoploid white clover (*Trifolium repens* L.) Genet. Pol. 11: 249-256.

Navalikhina N.K. (1973). Genetic bases of tetraploid clover breeding. Naukowa Dumka, Kijew: 135.

Poddubnaja-Arnoldi W.A. (1976). Citoembriologia pokrytosiemiennych rastienij. Nauka, Moskva: 507.

Rodkiewicz B. (1973). Embriologia roślin kwiatowych. PWN, Warszawa: 285.