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CYTOGENETIC RESEARCH ON HEXAPLOID ALFALFA, *MEDICAGO SATIVA* L.

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INTRODUCTION

In the genus *Medicago* only two hexaploid species ($2n=48$) are known, i. e. *M. cancellata* M. B. and *M. saxatilis* M. B. (LESINS 1959; LESINS and LESINS 1963), both perennial and native to the south-eastern part of the European U.S.S.R.. Only a few cases of 6x plants spontaneously occurring in natural tetraploid populations of *M. sativa* L. have been reported (DAVIES 1960; NIKOLOV 1962; BINGHAM and BINEK 1969; BINGHAM 1972); many hexaploids have been artificially obtained by crossing colchicine-induced octoploids (8x) with tetraploids (4x) (JULIÉN 1944; ARMSTRONG 1954; LESINS 1961).

The present paper describes the cytology and behaviour of a white-flowered hexaploid plant of *M. sativa*, occasionally found in a tetraploid population, which had been used in a programme of interspecific hybridization. In this programme several tetraploid plants of *M. sativa* with white flowers were crossed with the yellow-flowered *M. falcata*. Many seeds were obtained as expected, as the two species have the same chromosome number. Only one exception was observed when a particular plant of *M. sativa* was used: this plant produced only 5% of viable seeds when used as the mother plant and 7.9% in the reciprocal combination. The cytological analysis revealed that this plant had $2n=48$ chromosomes instead of $2n=32$ and its morphology and cytology were examined in detail.

Its significance in relation to the origin and evolution of *M. sativa* and

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its potential value for cytogenetic and breeding studies have also been considered.

MATERIALS AND METHODS

The material used was the white-flowered hexaploid plant of *M. sativa* plus some alfalfa tetraploid plants as control.

Root-tips, from rooted-cuttings in sand, were pretreated in saturated mono-bromo-naphtalene for 5 hours, then fixed in Carnoy's solution (3:1) and stained with the Feulgen method. Squash preparations were made in 50% acetic-acid. Total chromosome length (μ) was measured using a micrometer ocular. Ten metaphases for both the control and the hexaploid plant were chosen in which the chromosomes were well separated and reasonably straight to minimize inaccuracies in measurement. Chromosome pairing was studied in pollen mother cells after bud fixation in Carnoy's solution (3:1) and staining with acetic-carmine. For optimum results one or two drops of ferric-acetate were added.

Pollen grains were stained with acetic-carmine and pollen viability was expressed as percentage of well formed and stained grains on a total of 2000

Fig. 1. — Inflorescences of a white-flowered hexaploid (left) and tetraploid (right) plants of *Medicago sativa*.

for each sample (4x and 6x). Using the same preparations pollen diameters were measured with a micrometer ocular and expressed in μ as an average of 800 grains.

OBSERVATIONS AND DISCUSSION

The hexaploid plant resembled *M. sativa* plants of the original population except that all vegetative parts (leaves, stems, inflorescences) were larger; flowers were especially large, up to 13 mm in length, normally developed and numerous in each inflorescence (Fig. 1).

The chromosome number was determined on several metaphases in which monobromo-naphtalene pretreatment caused the chromosomes to be well separated and easily recognizable. All metaphase nuclei counted had 48 chromosomes (Fig. 2) including six with satellites (Figs. 3 a and b). The chromosomes, as in cultivated alfalfa were small and very similar in size and morphology. Most chromosomes had a sub-median centromere.

The six satellited chromosomes showed a constant length. Because of the great number of chromosomes, their small size and the difficulty in determining the exact position of the centromere, it was not possible to draw an

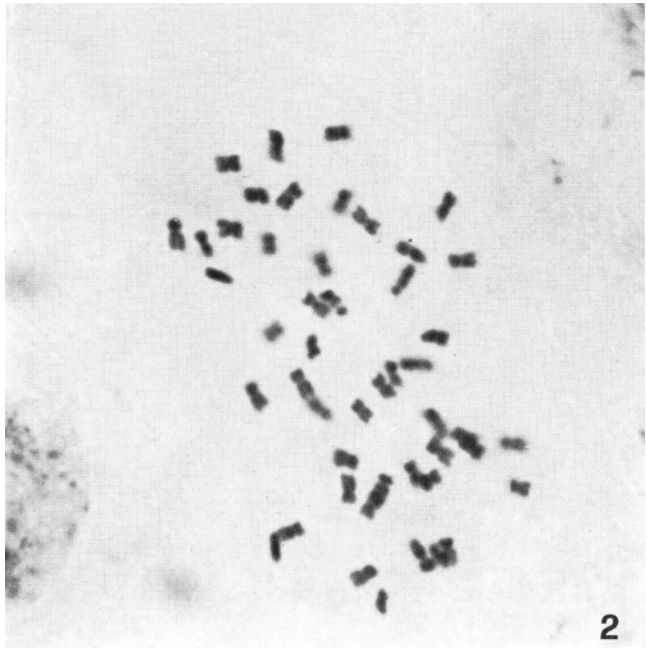


Fig. 2. — Root-tip cell with 48 chromosomes.

idiogram of the hexaploid plant on the basis of the arm length ratio, which is known to be constant.

However, for each chromosome the average total length was calculated on ten metaphases and compared with the chromosome length of the tetraploid

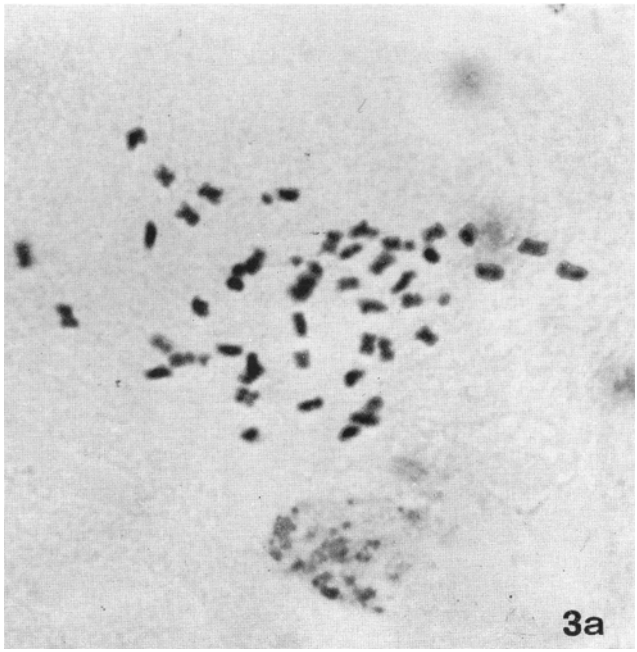


Fig. 3a. — Root-tip metaphase with 48 chromosomes three of which have satellites.
Fig. 3b. — Drawing of the same metaphase illustrating the six satellited chromosomes (black).

TABLE 1

Total chromosome length and percentage of pollen stainability in tetraploid and hexaploid alfalfa.

	4x	6x
Total chromosome length (μ)	2.08-2.83	1.34-2.25
% of stainable pollen	80.0	81.0

alfalfa calculated on the same number of metaphases (Table 1). The root-tips of the hexaploid and the tetraploid plants were pretreated in the same way, so that total lengths though not representing the true chromosome size, can be comparable in both materials. The chromosome length of the tetraploid varies between μ 2.08 and μ 2.83 while that of the hexaploid goes from μ 1.34 to μ 2.25. The data from BUSS and CLEVELAND (1968), who found that the length of the mitotic chromosomes of diploid alfalfa ($2n=16$) in 8-oxyquinoline pretreated root-tips, ranged from μ 3.5 to μ 4.5, suggest that in one species the chromosome length decreases as the ploidy level increases and that the reduction in length is much more substantial between the diploid and the tetraploid level than between the tetraploid and the hexaploid level.

GILLIES and LESINS (1971) observed the same pattern of length reduction of the chromosomes in connection with the increasing degree of ploidy in pachytene chromosomes of *M. sativa*. The variations found by the Authors in the chromosome length between diploids and tetraploids and between tetraploids and hexaploids are roughly the same as those observed in this study on mitotic chromosomes. There is certainly a precise link between the increase of the ploidy degree and the reduction of chromosome length. It would seem that the reason for such a reduction is to be ascribed directly and exclusively to the greater contraction of the chromosomes. However the exact reason which explains the effect of ploidy on chromosome contraction is still unknown *.

The results of the meiotic analysis carried out on the pollen mother cells, on a total of 245 cells, are reported in Table 2. Percentages were calculated on the total of the cells examined during each single phase. The percentage of pachytenes and diakinesis with one or two quadrivalents but more frequently

* The somatic chromosome measurements might be influenced by different cell cycle times in plants of different ploidy levels. It is to be noted however that GILLIES and LESINS (1971) obtained similar results when they measured pachytene chromosomes in alfalfa. Additionally PEGINGTON and REES (1970) working on somatic chromosomes in wheat, showed that the reduction in chromosome length with the increase in ploidy level, was due to increased metaphase coiling of the chromosomes. The present results, then, seem to be in agreement with previous observations by other Authors.

TABLE 2

Meiotic behaviour of a hexaploid plant of Medicago sativa L.. (percentages of the total number of cells observed in each stage).

First division									Second division	
Pachytene		Diakinesis		Metaphase 1			Anaphase 1		Anaphase 2	
% with II	% with 1-2 IV	% with II	% with 1-2 IV	% with 1-4 I	% with II	% with 1 IV	% with lagging chrom. 1-4	% without lagging chrom.	% with lagging chrom. 1-4	% without lagging chrom.
55.5	44.5	25.0	75.0	56.4	37.2	6.4	26.5	73.5	60.0	40.0

I = univalents, II = bivalents, IV = quadrivalents

with two, is rather high. We could never observe more than two quadrivalents per cell and in most diakinesis one quadrivalent associated with the nucleolus was present (Fig. 4); therefore the nucleolus organizer chromosome would be part of the quadrivalent. Bivalent chromosome association was by far the most common in all the phases observed and averaged about 20-24 bivalents per cell. Univalents occurred in 56.4% of the metaphase I cells and generally there were two or more of them per cell (Figs. 5 and 6). This percentage of

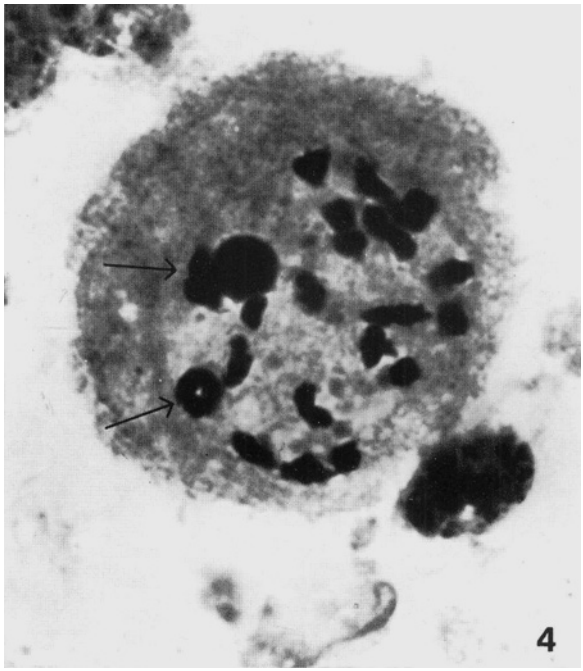
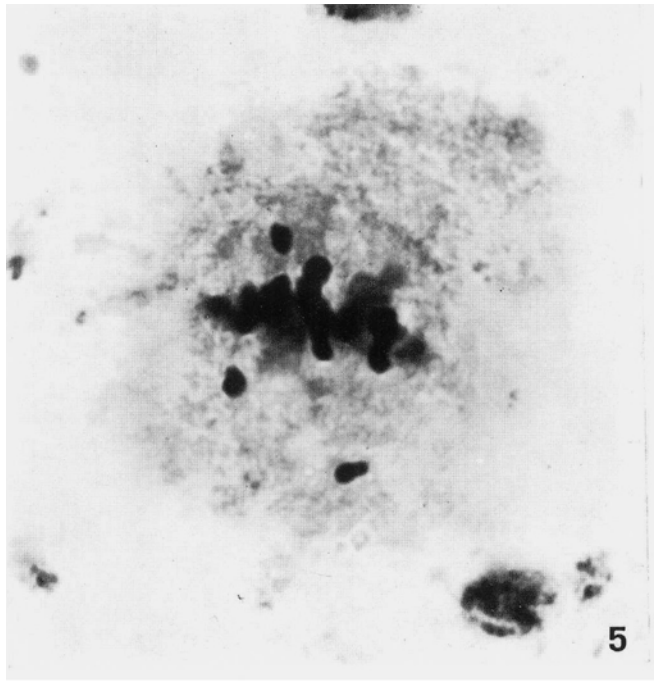


Fig. 4. — Diakinesis with 20 bivalents and 2 quadrivalents (arrows).

univalents is higher (56.4%) than that reported both by BINGHAM and BINEK (1969) in two hexaploid plants (41.3%) and by JULÉN (1944). However it appears from the literature that different hexaploids show a rather variable cytological behaviour. 26.5% of anaphases I were found with laggards varying in number from 1 to 4 (Fig. 7). Univalents tended to divide at the end of the first meiotic division and in turn produced lagging chromosomes in anaphase and telophase II. In fact observations resulted in 60% of anaphases II having from 1 to 4 laggards (Fig. 8), with a higher frequency of cells with 3 or 4 lagging chromosomes; three telophases II with a chromatinic bridge were also observed.

Rod bivalents were quite common, which would seem to mean that



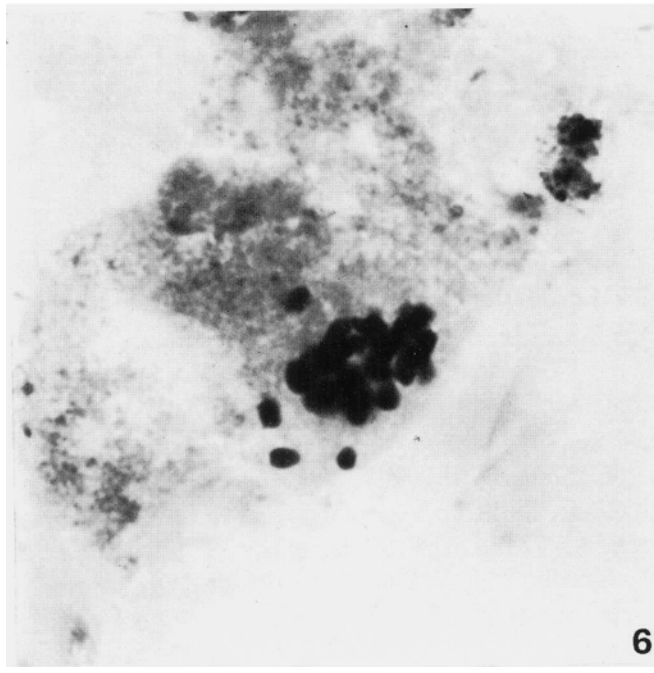
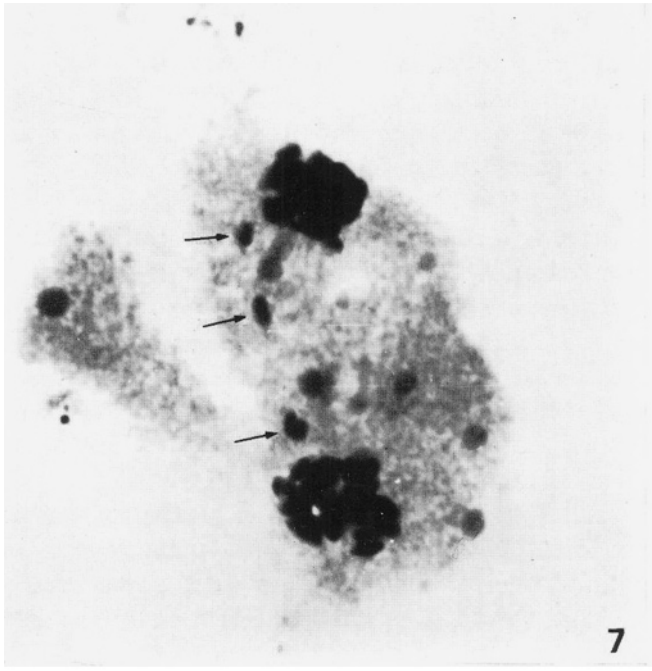


Fig. 5. — Metaphase I with three univalents.
Fig. 6. — Metaphase I with four univalents.



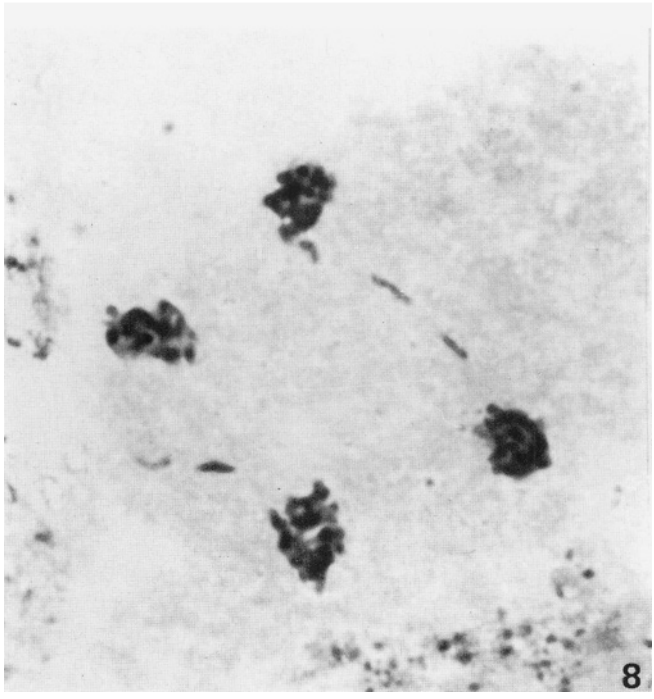


Fig. 7. — Anaphase I with three lagging chromosomes.
Fig. 8. — Anaphase II with some lagging chromosomes.

chiasmata are probably restricted to one chromosome arm. This agrees with the observations of BINGHAM and BINEK (1969), and might explain the predominant bivalent association in alfalfa at all ploidy levels and the occurrence of univalents in 4x, 6x and higher polyploids. It is possible to observe from table 2 that chromosome pairing disorders, namely univalents occurrence, appears at a certain moment of the meiotic cycle (metaphase I). Chromosome stickiness was also present in some metaphases and anaphases I. All the meiotic disorders observed, in particular univalents which produce nuclei with different chromosome numbers, might explain, at least partially, the almost complete sterility of the hexaploid plant. Pollen viability expressed as percentage of well formed and stained pollen was 81% in the hexaploid plant and 80% in tetraploid alfalfa used as control. Pollen grain diameters are reported in Fig. 9. The graph represents the diameter distribution in the hexaploid and tetraploid plant. The graph shows that the variability is much higher in 6x than in 4x plant: in fact, the variance estimates were 47.6 and 4.4 respectively,while the means were almost the same (μ 33.2) in 6x and (μ 33.4) in 4x. We attempted to classify the pollen grains of the hexaploid plant in two classes: the smaller one (less than 30μ) and the larger one (more than 30 μ). The means of the two classes were obviously very different (25.5 μ and 40 μ, respectively) and the variances were 10.3 for the smaller and 8.3 for the larger class: both much higher than in the tetraploid plant (4.4). The great pollen disformity in 6x compared with 4x is illustrated in Figs. 10a and b.

From the above considerations it can be observed that the class of pollen grains of the same size as the control is scarsely represented, while classes of smaller or larger size grains are much more frequent. This leads us to think that a large number of unbalanced gametes occur in the hexaploid and that such gametes, combined with meiotic disorders, might explain the very low fertility of the plant.

Crosses between the hexaploid plant and different *M. falcata* tetraploid plants produced, as reported before, very little seed. The 15 seeds obtained were sown but only 3 of these, from cross *M. falcata* x *M. sativa* germinated and developed normally. Thus three F_1 plants were obtained, whose chromosome number was found to be $2n=32$. Such a result is in agreement with the finding of BINGHAM and BINEK (1969) in 4x x 6x crosses. The tetraploid plants may have arisen from the union of gametes with 16 chromosomes from the hexaploid and gametes with 16 chromosomes from the tetraploid *M. falcata.* Therefore it can be assumed that the hexaploid produces gametes with a variable number of chromosomes and that there is apparently an advantage for gametes with 16 chromosomes or at least with a number of chromosomes very close to 16, even if this does not

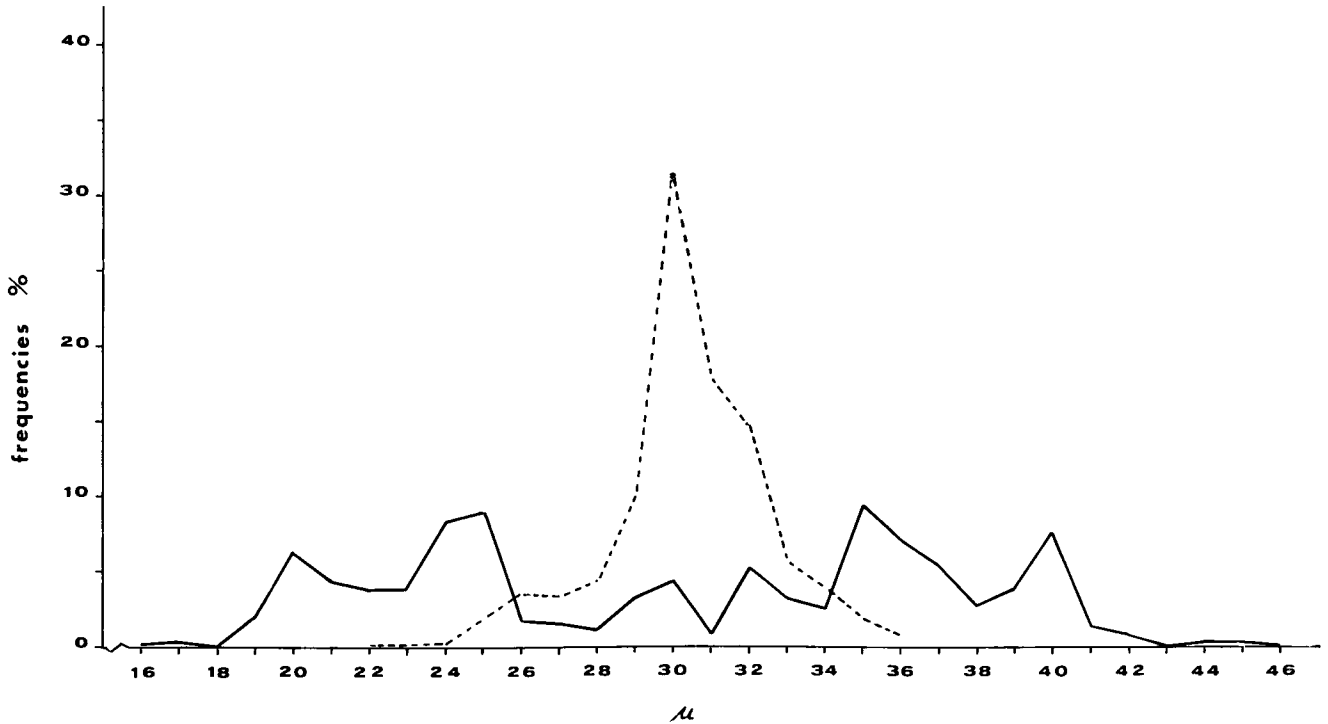


Fig. 9. — Diameters (μ) distributions of pollen grains in a tetraploid (- - - - -) and a hexaploid (———) plant of *Medicago sativa* L.

indicate that the frequency of aneuploid gametes is higher in the hexaploid than in the tetraploid. In fact it is known from previous studies (LEDINGHAM 1940) that the endosperm development in alfalfa depends on the ratio between the chromosome number of the female gametes and that of the male gametes, and that the gametes with a chromosome number close to that of the tetraploid appear to have a selective advantage.

An interesting point concerns the possible origin of the hexaploid plant. Three hypothesis can be formulated: 1) the union of normal reduced and occasional unreduced gametes from tetraploid parents; 2) a spontaneous somatic doubling in a triploid originated from 2x x 4x crosses and 3) the union of unreduced gametes formed by spontaneous octoploids and normal reduced gametes ($n=16$).

However the first hypothesis seems to be the most likely because the data of BINGHAM (1972) indicate that no spontaneous somatic doubling was observed among the cultivated and wild plants examined. In fact BINGHAM (1969) had previously obtained hexaploids either by the union of unreduced ($n=32$) and normal reduced ($n=16$) gametes, or by 3x x 6x crosses through production of unreduced triploid gametes.

The hexaploid plant was selfed by tripping 1000 flowers: only two pods and one seed were obtained, therefore we can say that this plant is almost completely sterile. This result is in accordance with that found by NIKOLOV (1962), who attributed sterility of the hexaploid plant studied to the embryosacs degeneration and to the meiotic disorders observed. On the contrary the hexaploid plants of the variety « Saranac » studied by BINGHAM and BINEK (1969) were both self and cross fertile. Some crosses between this plant and several alfalfa plants at different ploidy levels, are now in progress in order to clarify the causes of sterility of the hexaploid plants.

The observations of this study on chromosome homology and quadrivalent frequency, in agreement with most cytogenetic studies on *M. sativa*, support the hypothesis of the autotetraploid origin of alfalfa. Naturally further investigations will be required on other hexaploid material and on plants obtained through interploid crosses. Various crosses are being carried out between the hexaploid plant and *M. sativa* plants with different chromosome numbers, in order to check fertility at the hexaploid level and see whether higher ploidy levels are as stable as the tetraploids in their genetic behaviour.

Therefore the constitution of a hexaploid population could have some practical interest for alfalfa productivity and nutritional value tests, even at levels higher than the tetraploid. In fact from his experiments on spaced plants of *M. Sativa* varieties with different chromosome numbers (4x, 6x and 8x), DAVIES (1960) reached the conclusion that so far as productivity was concern-

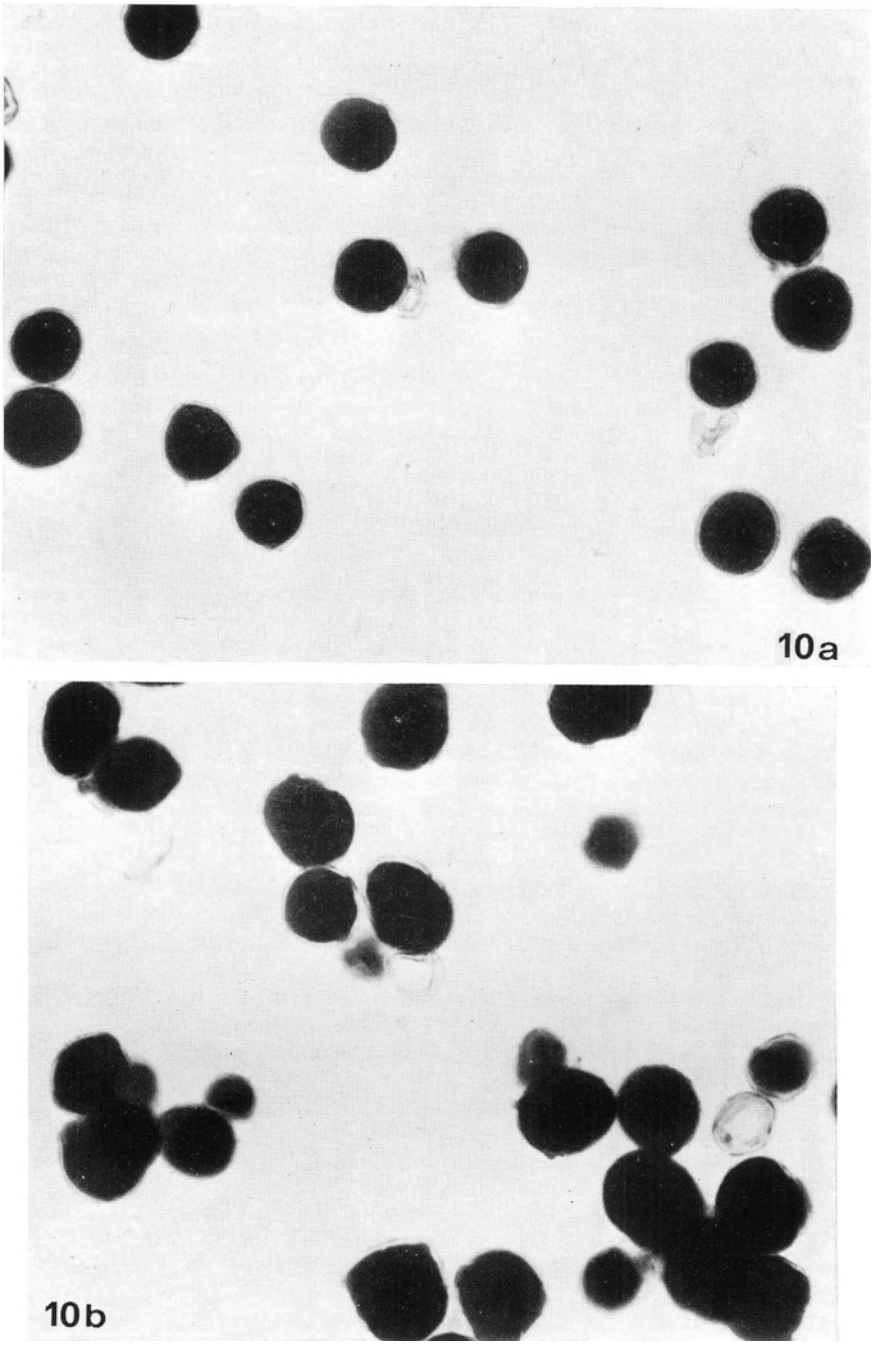


Fig. 10. — Tetraploid (a) and hexaploid (b) pollen grains.

ed the most promising plants were the hexaploids in that they were very leafy and resistant to some diseases.

Similarly LESINS *et al.* (1969) after comparing seed productivity in a few hexaploid plants of *M. sativa* versus plants of the Grimm variety, found that seed production was significantly higher than that of the Grimm variety for two consecutive years. The hexaploids might also be used for cytogenetic studies in generating aneuploids or individuals even with a great number of chromosomes by carrying out interploid crosses.

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SUMMARY

A spontaneously occurring hexaploid plant ($2n=6x=48$) was found among white-flowered tetraploid plants of *Medicago sativa*.

This plant was studied from a morphological and cytological point of view. All the metaphases observed showed 48 chromosomes, six of which with large satellites. Total chromosome length ranged from μ 1.34 to μ 2.25 whereas that of tetraploid ranged from μ 2.08 to μ 2.83. Most diakinesis showed bivalents and 1 or, more frequently, 2 quadrivalents. Two or more univalents were observed in 56.4% of metaphases I and lagging chromosomes, varying in number from 1 to 4, were observed in 60% of anaphases II. Hexaploid pollen stainability (81%) was the same as in the tetraploid plants used as control (80%). The pollen grains were very variable in diameter (μ) and therefore two classes were distinguished: a smaller (diameter μ 25.5) and a larger one (diameter μ 40.0). Such variability probably means that the hexaploid plant produces many unbalanced gametes which, in association with meiotic disorders may explain the very low fertility of the hexaploid plant. The importance of hexaploid for cytogenetic researches, for agronomic studies and for its significance in the evolution of *Medicago sativa* are discussed.