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Use of anaphase I separations and the number of micronuclei in tetrads for selection of fertility in ryegrass

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ABSTRACT

The characters causing low seed-setting rates in induced tetraploid *Lolium perenne* L. were investigated. The objective of this study was to determine whether these characters could be used as criteria in the selection of regular meiosis and high seed-setting plants.

The seeds of C_1 and C_2 generations of artificially obtained tetraploid perennial ryegrass were used. Cytological studies were done on the root tips and spike samples. A significant positive correlation was found between the frequency of cells showing regular anaphase I and high seed-setting rates. As a result, it was decided that this normal separation in anaphase I could be used as a selection criterion for seed set. The highly significant negative correlation that was found between the number of micronuclei and seed setting showed that this character could also be used as a selection criterion when a large population is under consideration. It was necessary to isolate aneuploids from euploid plants to increase fertility.

INTRODUCTION

After the report of Blakeslee (1937) on the efficacy of colchicine in the induction of chromosomal reduplication in plants, this chemical has been used successfully in a great number of plant species. It should be possible, by proper timing of the duration of treatment, to change a whole tissue from diploid to polyploid.

Perennial ryegrass (*Lolium perenne* L.) is a natural diploid (2n = 14) and an allogamous species. In autotetraploid perennial ryegrass percentage of dry matter was lower than their diploids, whereas hay yield, digestibility, and food quality were higher in autotetraploids (Ahloowalia, 1971a). The first autotetraploid plants of *Lolium perenne* were produced by Myers (1939) using the colchicine method. Myers (1945) was also the first to study meiotic behavior in induced autotetraploid ryegrass. Later, the results of cytological studies were published by Wit (1959), Ahloowalia (1967), Crowley and Rees (1968), De Roo (1968), and Sağsöz (1974).

Reduced fertility of induced autotetraploids as compared with the diploid original populations is a common feature of autotetraploids (Sağsöz, 1974; Akgün et al., 1999). Meiosis with a high frequency of irregularities may result in a fairly high frequency of aneuploid gametes and aneuploid progeny.

The objectives of this study were to investigate the mitotic chromosome number, the cytological causes of irregular meiosis and low seed set in C_1 and C_2 generations of induced tetraploid ryegrass (*Lolium perenne* L.), and to investigate the possibility of using some cytological features such as regular anaphase I and tetrad without micronuclei as the selection criteria in regular meiosis and high seed set.

MATERIAL AND METHODS

The seeds of C_1 and C_2 generations of the induced tetraploid ryegrass obtained from Sağsöz (1974) were used in this experiment. A diploid ryegrass cultivar from

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Eskişehir, Turkey was taken to Atatürk University and, after selection, given the registration number 6/13. Colchicine was used to obtain autotetraploid plants of 6/13. Cytological studies were conducted on the C_1 and the C_2 generations.

One seed from each pot was sown and root-tip samples for mitotic chromosome counting were taken from growing seedlings showing tetraploid characteristics (width, length, and thickness of leaves). Thus, chromosome counting was done in 26 C_1 plants and 90 C_2 plants. Five to eight root-tips were selected from each plant and cut to 1.0–1.5-cm lengths. The root-tips were pretreated in 1-bromo-napthalene saturated with water for 3 h at 24 °C; fixed in Carnoy's solution (ethanol/acetic acid/chloroform, 6:3:1); stained by the Feulgen method after hydrolyzing for 10 min in 1 N HCl at 60 °C; and squashed in 45% acetic acid (Sağsöz, 1976).

After chromosome counting, plants were subjected to vernalization at 0 °C for 12 weeks to induce generative development. The plants of C_1 and C_2 generations and control (diploid) were grown in a growth chamber illuminated for 16 h. Day and night temperatures were 25 ± 2 and 20 ± 2 °C, respectively. In meiotic studies, spikes were fixed in Carnoy's solution. After 24 h, the spikes were transferred to 70% alcohol and stored at 4–5 °C. For staining and spear preparations 2% aceto-carmine methods were used (Sağsöz, 1974). The diploid plants (control) were not used for meiotic studies.

The seed set was determined in 4 diploids (control), 13 C_1 plants, and 30 C_2 plants. When determining seed sets, spikelets of top and bottom of spike were not taken into consideration (Fedak, 1975). The percentage of seed set was calculated as follows:

$$\text{Percentage of seed set} = \frac{\text{Number of seeds in spike} \times 100}{\text{Total floret number in spike}}$$

The correlations between seed set and regular anaphase I cells, and seed set and the number of micronuclei in tetrads were calculated.

RESULTS

Chromosome number in the autotetraploid C_1 and C_2 populations

As can be seen in Table 1, in the C_1 generation 30.8% diploid, 38.5% tetraploid, and 30.7% aneuploid plants were obtained. Also, in the C_2 generation 31.1% diploid, 45.6% tetraploid, and 23.3% aneuploid plants were obtained. In the C_1 generation, the number of chromosomes ranged from 2n = 25 to 2n = 29. In the C_2 generation, in addition to the C_1 generation chromosome numbers, two plants with 2n = 30 (Fig. 1 a–d) and one plant with 2n = 34 were observed.

The stage of anaphase I

Although the determination of anaphase distribution was labor intensive at this stage, the chromosomes were easily observed and counted. The stage of anaphase I could be examined in 13 C_1 plants and 38 C_2 plants and the data are given in Table 2.

In euploids (2n = 28) of the C_1 generation, the percentages of anaphase I showing regular segregation (14:14) and irregular segregation (13:15, 12:16) were 41.5% and 21.7%, respectively. In addition, 35.9% and 0.9% of anaphase I had lagging chromosomes and/or chromatids and a bridge, respectively. In aneuploids with 27 and 29 chromosomes of C_1 generation, the percentages of regular anaphase I cells were 40.5% (13:14 chromosome segregation) and 42.1% (14:15 chromosome segregation), respectively (Table 2).

In tetraploids (2n = 28) of C_2 generation, the percentage of anaphase I with regular segregation (14:14), irregular segregation (13:15, 12:16, 11:17, etc.), lagging chromosomes, and a bridge were 40.5, 17.3, 39.2, and 2.9%, respectively (Fig. 2a–c; Table 2). The aneuploids with 26 and 30 chromosomes had fairly high abnormal anaphase I segregations (uneven chromosome separations, lagging chromosomes and bridges).

In the euploids of C_1 and C_2 generations, the percentages of regular anaphase I cells (41.5% and 40.5%,

Table 1
Chromosome number and frequency of the different cytotypes in the C_1 and C_2 generations

		Somatic chromosome number								
		Diploid	Tetraploid	Aneuploids						
Generation	Plant	(2n = 14)	(2n = 28)	2n = 25	2n = 26	2n = 27	2n = 29	2n = 30	2n = 34	Total
C_1	number	8	10	1	2	3	2	—	—	8
	%	30.8	38.5	3.8	7.7	11.5	7.7	—	—	30.7
C_2	number	28	41	1	2	7	8	2	1	21
	%	31.1	45.6	1.1	2.2	7.8	8.9	2.2	1.1	23.3

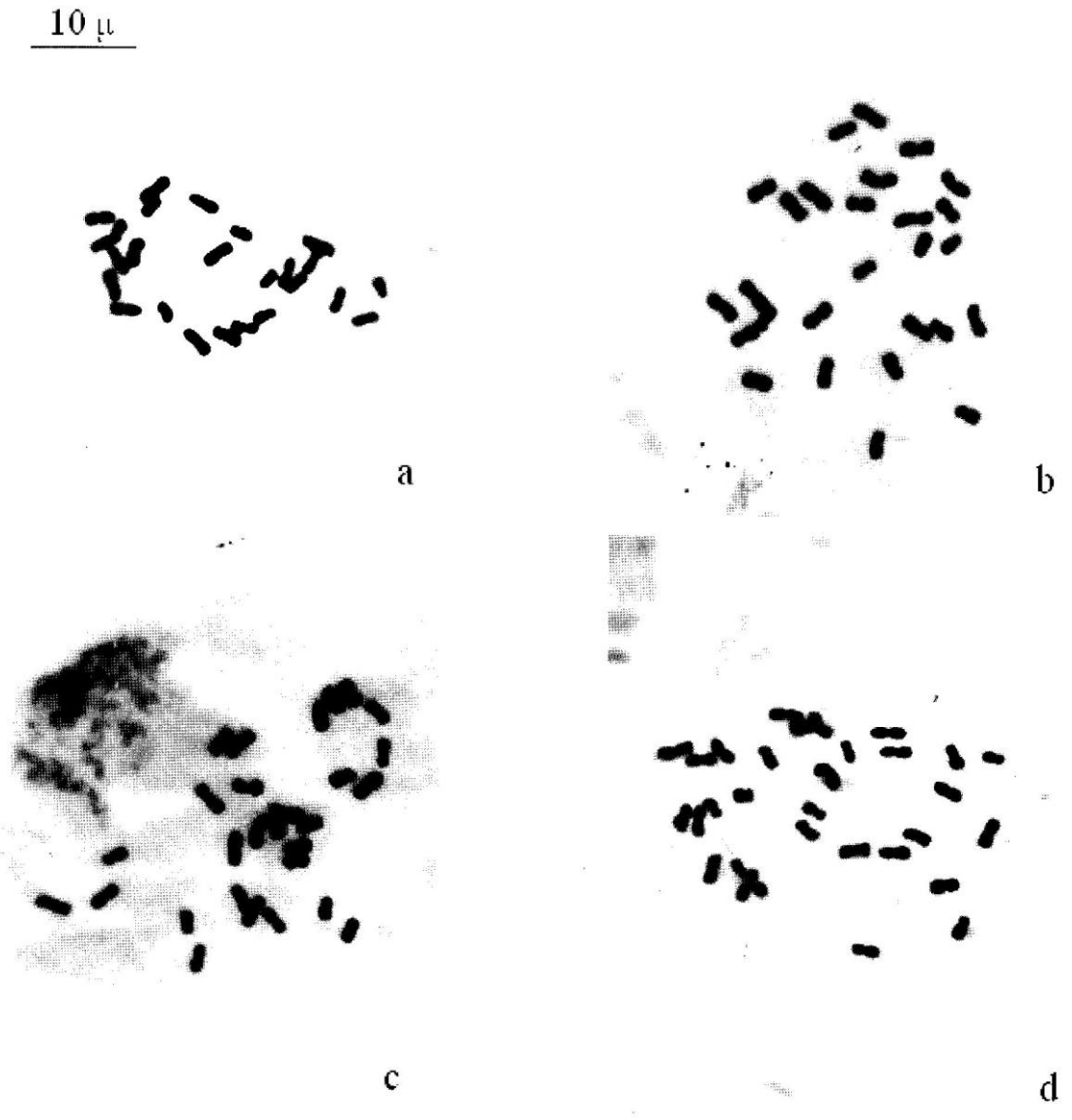

Fig. 1. Metaphase chromosomes in root-tip cells of autotetraploid *Lolium perenne* L. in C_2 generation. a. 2n = 25, b. 2n = 27, c. 2n = 28, d. 2n = 30.

respectively) were close to one another (Table 2). Most cells with abnormal anaphase I segregations had 1–7 lagging chromosomes and chromatids, which were formed from division of univalents.

The stage of tetrad

The tetrad stage was examined in 13 C_1 plants and 32 C_2 plants. To determine the number of micronuclei per tetrad (M/Q) and the percentages of tetrads with and without micronuclei, 100 tetrads in each plant were examined. The results are presented in Table 3. In euploids of C_1 and C_2 generations, M/Q was 0.9 and 1.1, respectively, while the percentage of tetrads without micronuclei was 60.0 and 53.6%, respectively (Table 3 and Fig. 2d). The highest number of micronuclei per tetrad (3.4) was determined in an aneuploid plant with

Table 2
Percentage of anaphase I having regular and irregular chromosome distributions, lagging chromosomes, and/or chromatids and bridges

No. of Chromo. (2n)	C_1 Generation						C_2 Generation					
	No. of plants	No. of PMC	Reg., %	Irreg., %	Lag., %	Bridge, %	No. of plants	No. of PMC	Reg., %	Irreg., %	Lag., %	Bridge, %
28	8	106	41.5	21.70	35.9	0.9	22	306	40.5	17.3	39.2	2.9
25	—	—	—	—	—	—	1	14	28.6	7.1	64.3	—
26	—	—	—	—	—	—	1	13	7.7	23.1	69.2	—
27	3	37	40.5	5.4	48.7	5.4	7	84	39.3	7.1	52.4	1.2
29	2	19	42.1	42.1	10.5	5.3	6	70	31.4	11.4	48.6	8.6
30	—	—	—	—	—	—	1	12	16.7	41.7	25.0	16.7
Mean or Total	13	162	41.4	23.1	31.7	3.8	38	499	27.4	18.0	49.8	4.9

34 chromosomes, followed by an aneuploid plant with 25 chromosomes (3.2). On the other hand, the aneuploid plant with 25 chromosomes had the lowest percentage of tetrad without micronuclei (6.0%) followed by aneuploids with 2n = 26 (7.0%) and 2n = 34 (10.9%) chromosomes.

Percentage of seed set

Thirteen plants of C_1 population, which were used for the meiotic studies, were grown in a growth chamber. Two growth chambers were used, one chamber for plants with 28 chromosomes, the second one for aneuploids and diploids. Thus, negative effects of diploids and aneuploids on seed sets of euploids were eliminated. The highest seed set in the C_1 population (41.3%) was determined in euploids. In aneuploids with 2n = 27 and 2n = 29 chromosomes of C_1 generation, seed sets were 29.0% and 31.9%, respectively (Table 4).

Thirty plants of C_2 generation used in meiotic studies were grown in a growth chamber. Euploid C_2 plants could not be separated from aneuploids. The highest seed sets in the C_2 population, similar to the C_1 population, were determined in euploids (32.3%). This was followed by aneuploids with 2n = 27 (22.4%) and 2n = 29 chromosomes (21.3%), respectively. The seed set of other aneuploids was low. As expected, the control diploids always had the highest seed sets (Table 4).

The correlations between some cytological characteristics and seed set

The correlations were calculated in the euploid plants of both generations. A significant positive correlation ($p < 0.01$) was found between the frequency of regular anaphase I and high seed set in C_1 (0.921) and C_2 (0.866) generations. The correlation between seed set and the number of micronuclei in tetrads in C_1 and C_2 generations was –0.929 ($p < 0.01$) and –0.608 ($p < 0.01$), respectively. A significant positive correlation ($p < 0.01$) was also found between the frequencies of anaphase I with lagging chromosomes and the frequency of the micronuclei in tetrads in C_1 (0.869) and C_2 (0.655) generations.

DISCUSSION

High aneuploidy frequency in autoploid species affects significantly seed production. The tolerance of species to aneuploidy differs. No aneuploidy has been observed in *Lolium* as they have no functional aneuploid gametes (Barclay and Armstrong, 1966). It has been reported that aneuploidy frequency in *Lolium* species was high (6–59%) (Easton, 1973; Simonsen, 1973; Sağsöz et al., 1998). This high frequency can be explained as either the existence of higher frequency of aneuploid gametes or higher viability of gametes. Aneuploidy frequency shows variability based on species as well as population having the same species (Klinga, 1986a). Genotype × environment interaction can also produce aneuploid plants (Romel, 1963). Environmental factors and amount of pollen produced can influence the competition within aneuploid gametes or zygotes. When there are sufficient amounts of pollen and necessary optimum conditions, selection among pollens happens to be in favor of euploids (Elgersma, 1993). If there is no or little competition, aneuploid gametes and zygotes can produce a viable seed set (Ellerström and Sjödin, 1974). In addition, their genetic backgrounds can affect this competition.

In addition to the many advantages of artificial autopolyploids, they have some important problems such as unbalanced gene combination or failure of cytological and physiological processes, as well as having

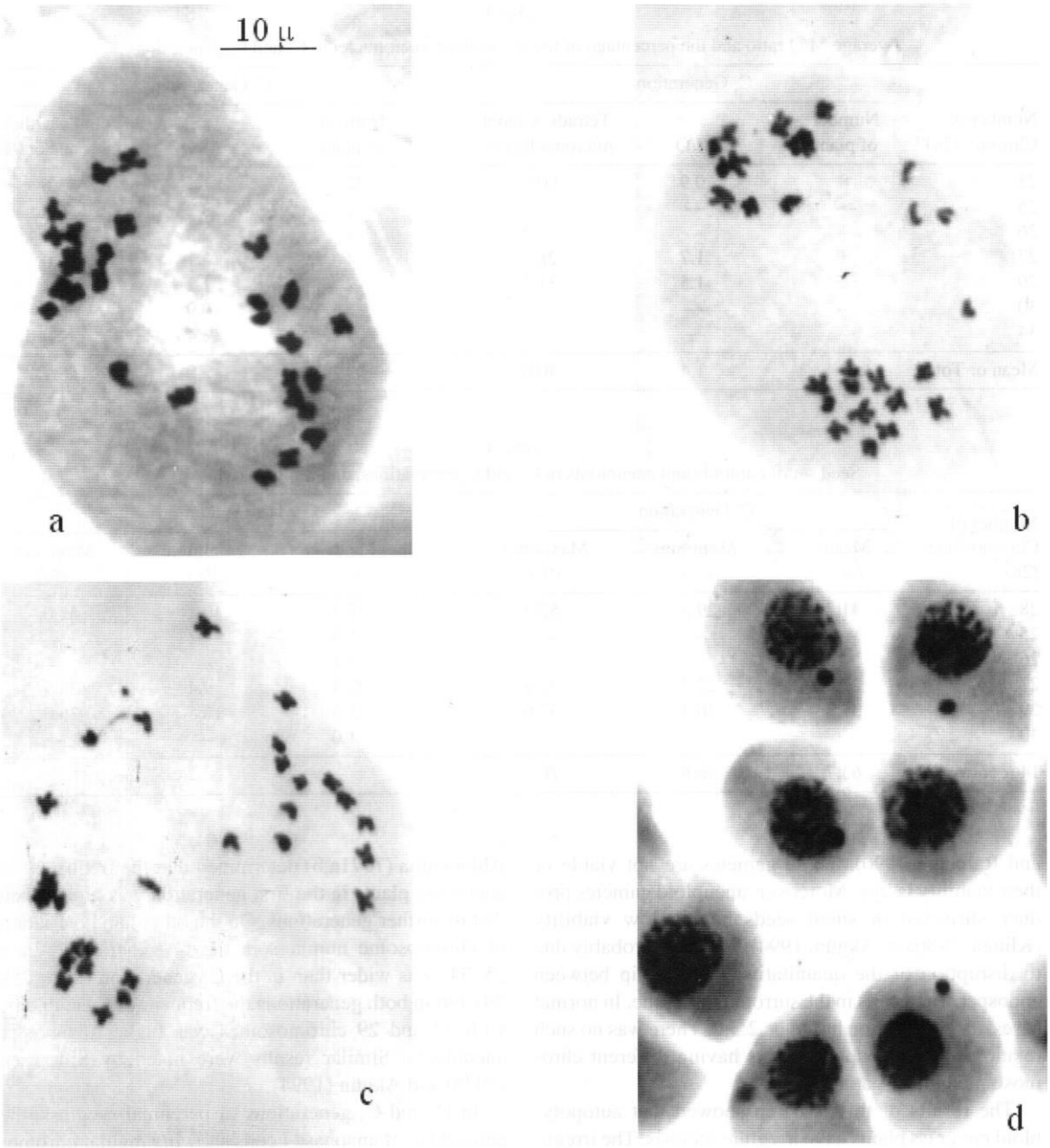

Fig. 2. Anaphase I cells and tetrads in autotetraploid and aneuploid ryegrass. a. 13:13 distribution and 2 lagging chromosomes (2n = 28), b. 12:12 distribution and 2 lagging chromosomes and 2 splitting chromatids (2n = 26), c. dicentric bridge and acentric fragment in anaphase I, d. tetrads with micronuclei.

aneuploid plants resulting from aneuploid gametes. It was claimed that low seed set was caused by aneuploids (Klinga, 1986b) or allele incompatibility (Fearson et al., 1984) in tetraploids. Problems originating from incompatibility can be solved easily. However, it is difficult to solve problems related to the chromosome pairing system (Wilkins, 1991). Bivalent pairing as well as trivalent and quatrivalent pairing can also be seen in autotetraploid plants based on increases in number of homolog chromosomes. The pairing schemes seen in metaphase I cause aneuploid gametes by means of chromosome laggings and irregular chromosomes separations (Evans

Table 3

Average M/Q ratio and the percentage of tetrads without micronuclei in C_1 and C_2 generations

Number of Chromo. (2n)	C_1 Generation			C_2 Generation		
	Number of plant	M/Q	Tetrads without micronuclei (%)	Number of plant	M/Q	Tetrads without micronuclei (%)
28	8	0.9	60.0	22	1.1	53.6
25	—	—	—	1	3.2	6.0
26	—	—	—	1	2.3	7.0
27	3	1.7	28.4	4	2.1	29.0
29	2	1.5	31.7	2	1.2	36.8
30	—	—	—	1	0.9	53.3
34	—	—	—	1	3.4	10.9
Mean or Total	13	1.4	40.0	32	2.0	28.1

Table 4

Seed set of euploids and aneuploids in C_1 and C_2 generations, and diploids (control)

Number of Chromosome (2n)	C_1 Generation			C_2 Generation		
	Mean (%)	Minimum (%)	Maximum (%)	Mean (%)	Minimum (%)	Maximum (%)
28	41.3	29.2	52.4	32.3	10.1	56.0
25	—	—	—	5.3	—	—
26	—	—	—	2.1	—	—
27	29.0	25.7	32.9	22.4	9.4	28.9
29	31.9	26.3	37.6	21.3	16.5	26.0
30	—	—	—	1.0	—	—
14 (control)	63.7	56.8	70.7			

and Rahman, 1990). These gametes are not viable or their viability is low. Moreover, aneuploid gametes produce shriveled or small seeds having low viability (Klinga, 1986a,b; Akgün, 1994). This was probably due to disruption of the quantitative relationship between endosperm and cells in the surrounding tissue. In normal cases, this interaction has to be 2:3:2. There was no such relationship in aneuploid plants having different chromosome number gametes.

The results of this research showed that autopolyploid rye grass plants have irregular meiosis. The irregularities at meiosis may result in the formation of aneuploid gametes and progenies. There were three major types of meiotic irregularity in autotetraploid *Lolium perenne*; (1) unbalanced quadrivalent separation, (2) incomplete separation of chromosomes resulting in lagging and dividing univalents at anaphase I, and (3) high frequency of univalents that tended to lag and divide equally at anaphase I, resulting in micronuclei at telophases I and II.

The percentage of aneuploids among the C_1 generation was higher than that of the C_2 generation. Similarly, Ahloowalia (1971a,b) determined that the frequency of aneuploid plants in the first generation was higher than that of further generations. On the other hand, variation of chromosome numbers in the C_2 generation (2n = 25–34) was wider than in the C_1 generation (2n = 25–29), but in both generations the frequency of aneuploids with 27 and 29 chromosomes was higher than other aneuploids. Similar results were noted by Simonsen (1975) and Akgün (1994).

In C_1 and C_2 generations of perennial ryegrass, the percentage of anaphase I cells showing regular chromosome segregation was higher in euploids. In addition, percentages of regular anaphase I cells in aneuploids of C_1 generation were almost the same as percentages of regular anaphase I cells in euploids. It is assumed that this resulted from the anaphase I cells having 13:14 segregations in aneuploid with 2n = 27, and 14:15 segregations in aneuploid with 2n = 29 behaving as regular anaphase I. The percentages of regular anaphase I in the aneuploids with 27 and 29 chromosomes of C_2 generation were higher than those of other aneuploids of the same generation. On the other hand, the bridges observed

in this study could originate from paracentric inversions (Sybenga, 1992).

The number of micronuclei per tetrad in the euploid of the C_2 generation was higher than that of the C_1 generation. This may result from higher numbers of anaphase I lagging chromosomes and bridges in C_2. Indeed, a significant positive correlation was found between the frequency of anaphase I with lagging chromosomes and the frequency of the micronuclei in tetrads. Generally, the aneuploids of both generations had high M/Q.

As can be seen from Table 4, the seed set of C_1 plants was higher than that of C_2 plants. It is supposed that the low seed set of C_2 plants may have originated from aneuploids that were not isolated from the euploids. Similar results were noted by some researchers (Ellerström and Sjodin, 1966; Ahloowalia, 1967, 1971b; Aastveit, 1968; Mary et al., 1973; Simonsen, 1975).

The present study shows that in autotetraploid *Lolium perenne,* normal separations in anaphase I could be used as a useful selection criterion to decide about the regular or irregular meiosis. We found that the correlation between the frequency of the cells having normal anaphase distribution and high seed set was significant and positive in C_1 and C_2 generations. Our studies are in accord with the observations of Simonsen (1973), Fedak (1975), Akgün (1994), Tosun (1995), and Akgün et al., (1999).

Meiotic regularity has to be improved to increase the seed set due to the relationship between fertility and regular meiosis. Hence, this can prevent aneuploid gametes being an important problem for cultivar purity.

We suggest using tetrads (number of micronuclei) as a selection criterion when a large population is under consideration. It should be kept in mind that isolations of euploid plants from the aneuploids in allogam species would positively influence seed sets.

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